University of Alberta

Evaluation of the Protection Induced by a Monotherapy of Anti-LFA-1 Monoclonal Antibody and Co-transplantation of Neonatal Porcine Islets with Sertoli Cells

by

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ABSTRACT

Two major barriers to islet transplantation are the need for an unlimited source of donor tissue and a safer method of immunosuppression. These may be overcome by xenotransplantation of neonatal porcine islets (NPI) along with combined co-transplantation of neonatal porcine Sertoli cells (SC) and transient use of anti-LFA-1 monoclonal antibody (mAb). In this study we aimed to identify potential mechanisms responsible for prolonged NPI islet xenograft survival with our combination therapy.

Our data demonstrates that the combination of anti-LFA-1 mAb therapy along with the co-transplantation of SC is indeed highly efficacious in preventing NPI xenograft rejection as 20/27 treated mice achieved and maintained long-term graft survival. Although it appears that T regulatory cells are not solely responsible for maintaining long-term xenograft protection, they are likely important in establishing a T_H2 cell phenotype and sharing a role with secreted SC products, such as serpina3n, in prolonging NPI xenograft protection.

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LIST OF ABBREVIATIONS

Ab	Antibody
ABC	Avidin-binding complex
APC	Antigen presenting cells
ATP	Adenosine triphosphate
B6	C57BL/6
BTLA	B- and T- lymphocyte attenuator
BSA	Bovine serum albumin
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DAB	3,3-diaminobenzidinetetrahydrochloride
DM	Diabetes mellitus
FACS	Florescence activated cell sorter
FasL	Fas ligand
Foxp3	Forkhead box P3
GITR	glucocorticoid-induced tumor necrosis factor receptor-related protein
HBSS	Hank's Balanced Salt Solution
HLA	Human leukocyte antigen
IBMIR	Instant blood mediated inflammatory reaction
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LFA-1	Leukocyte function associated antigen-1
mAb	Monoclonal antibody
MAC	Membrane attack complex
MHC	Major histocompatibility complex
MIS	Müllerian inhibiting substance
NOD	Non-obese diabetic
NPI	Neonatal porcine islets
OCT	Optimum cutting temperature
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PERV	Porcine endogenous retrovirus
SC	Sertoli cells
Serpina3n	Serine proteinase inhibitor-3
STZ	Streptozotocin
TCR	T cell receptor
TGF-β	Transforming growth factor beta
T_H	T helper
TNF-α	Tumor necrosis factor alpha
T_R	T regulatory

CHAPTER 1

GENERAL INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) encompasses a common group of metabolic disorders, which share the similar quality of hyperglycemia[1]. Hyperglycemia may be a result of numerous contributing factors, but all diabetic states are considered a result of an inadequate supply of insulin and/or an inadequate response of the tissues towards the actions of insulin[1,2,3]. The patient's chronic inability to store glucose leads to an increased circulating glucose concentration, which is connected to abnormalities in carbohydrate, protein, and fat metabolism, as well as countless complications, including damage and failure in numerous organs such as the heart, kidneys, eyes, nerves, and blood vessels[2,3,4]. Persistent hyperglycemia is characterized by several symptoms, most notably polyuria and polydipsia[1,3]. Other symptoms include polyphagia, weight loss, fatigue, and blurred vision, while growth impairments and increased susceptibility to secondary infections may also affect patients having chronic hyperglycemia[1,3].

Uncontrolled diabetes may have acute and chronic consequences. Diabetic ketoacidosis and hyperglycemic hyperosmolar syndrome are two serious, acute complications associated with uncontrolled diabetes[3].

Chronic consequences of diabetes may include nephropathy leading to kidney failure, retinopathy with potential vision loss, peripheral neuropathy causing a need for amputation, and autonomic neuropathy causing cardiovascular, gastrointestinal, genitourinary symptoms, and sexual dysfunction[3]. Patients are also at an increased risk for atherosclerotic cardiovascular, cerebrovascular, and peripheral arterial disease[3].

1.1.1 Impact of Diabetes

1.1.1.1 Personal Burdens

The personal complications associated with diabetes is a large cause for concern, as patients must be responsible for daily measuring blood glucose levels, and may potentially be forced to take exogenous insulin daily in the form of injections[2]. Diabetics can be further limited in their lifestyle due to constant fluctuations in blood glucose associated with such things as food intake, levels of stress, and levels of activity[2]. Direct costs a diabetic patient can encounter for medication and supplies may reach up to $15,000 per year in Canada[5]. Diabetes can result in significant morbidity, and numerous secondary complications which can damage the patient physically, mentally, and psychologically[1,5,6]. These burdens, along with a decreased life expectancy, have the ability to drastically decrease a diabetic patient's quality of life[1,5].

1.1.1.2 Global Burdens

The number of people worldwide currently estimated to have diabetes is a stunning 285 million[5]. By the year 2030, that number is expected to reach 438 million[5]. In Canada alone, the number of people with diabetes or prediabetes has reached 9 million in 2011, with 10% of these patients affected by type 1 diabetes[5]. Diabetes is the fourth leading cause of death worldwide, responsible for approximately 3.8 million deaths per year[6,7,8]. In the United States, DM is the leading cause of lower extremity amputations, end-stage renal disease, and adult blindness[1]. Diabetes also has a major economic impact, evident by the projections that diabetes will cost the Canadian healthcare system $16.9 billion per year by 2020[5]. Global health care expenditures on diabetes prevention and treatment are estimated to exceed $490 billion US dollars by 2030[6].

1.1.2 Classification of Diabetes

The majority of diabetic patients fall under two major categories. As the understanding for the underlying pathophysiological mechanisms developed, so did the classifications (Table 1.1). Type 1 DM, formerly known as insulin-dependent DM or juvenile-onset DM, can also be split into subcategories based on the presence or absence of autoimmunity[2,7]. Type 1a is characterized by a T cell autoimmune attack on the β cells in the islets of Langerhans, leading to the total absence of insulin secretion[2,7]. Type 1b also includes patients who experience a lack of

insulin secretion due to an absence of β cells, however, it is idiopathic in origin, and may not have an immune-mediated etiology[2,7]. Type 2 DM, formerly known as non-insulin dependent DM, is characterized by insulin resistance in combination with reduced insulin secretion, along with an inadequate compensatory insulin secretory response by body tissues[2,7]. Although Type 2 DM is much more common, the clinical symptoms are generally less severe, as most patients do not require insulin injections for survival[2,3,4]. Increased age, obesity, and sedentary lifestyle have been found to increase the risk of incurring type 2 DM[1,2,4].

Another form of diabetes which occurs in pregnant females is gestational diabetes. Gestational diabetes is characterized by insulin resistance, and is found to affect approximately 2-5% of pregnancies[1,2]. Other forms of DM, often termed secondary diabetes, can result from or are related to another specific disease process or genetic disorder[2,7]. Secondary diabetes includes genetic defects in β cell function and insulin action, exocrine pancreatic diseases, endocrinopathies, drug or chemical induced diabetes, infections, genetic syndromes associated with diabetes (i.e. Klinefelter's syndrome), and other uncommon forms of immune-mediated diabetes[2,7].

Classifications of Diabetes

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)
   A. Immune mediated
   B. Idiopathic
II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)
   A. Genetic defects of β-cell function
      1. Chromosome 12, HNF-1α (MODY3)
      2. Chromosome 7, glucokinase (MODY2)
      3. Chromosome 20, HNF-4α (MODY1)
      4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
      5. Chromosome 17, HNF-1β (MODY5)
      6. Chromosome 2, *NeuroD1* (MODY6)
      7. Mitochondrial DNA
      8. Others
   B. Genetic defects in insulin action
      1. Type A insulin resistance
      2. Leprechaunism
      3. Rabson-Mendenhall syndrome
      4. Lipoatrophic diabetes
      5. Others
   C. Diseases of the exocrine pancreas
      1. Pancreatitis
      2. Trauma/pancreatectomy
      3. Neoplasia
      4. Cystic fibrosis
      5. Hemochromatosis
      6. Fibrocalculous pancreatopathy
      7. Others
   D. Endocrinopathies
      1. Acromegaly
      2. Cushing's syndrome
      3. Glucagonoma
      4. Pheochromocytoma
      5. Hyperthyroidism
      6. Somatostatinoma
      7. Aldosteronoma
      8. Others
   E. Drug- or chemical-induced
      1. Vacor
      2. Pentamidine
      3. Nicotinic acid
      4. Glucocorticoids
      5. Thyroid hormone
      6. Diazoxide
      7. β-adrenergic agonists
      8. Thiazides
      9. Dilantin
      10. α-Interferon
      11. Others
   F. Infections
      1. Congenital rubella
      2. Cytomegalovirus
      3. Others
   G. Uncommon forms of immune-mediated diabetes
      1. "Stiff-man" syndrome
      2. Anti-insulin receptor antibodies
      3. Others
   H. Other genetic syndromes sometimes associated with diabetes
      1. Down's syndrome
      2. Klinefelter's syndrome
      3. Turner's syndrome
      4. Wolfram's syndrome
      5. Friedreichs's ataxia
      6. Huntington's chorea
      7. Laurence-Moon-Biedl syndrome
      8. Myotonic dystrophy
      9. Porphyria
      10. Prader-Willi syndrome
      11. Others
III. Gestational diabetes mellitus (GDM)

Table 1.1: Etiological classifications of diabetes mellitus. Adapted from: Diagnosis and classification of diabetes mellitus. Diabetes Care 27 Suppl 1. S5 – S10 (2004).

1.2 TYPE 1 DM

Interestingly, type 1 DM is commonly characterized by destruction of beta cells within the islets of Langerhans, while the other cell types in the pancreas maintain their function[2]. Type 1 DM encompasses roughly 5-10% of diabetic patients[3]. The majority of these patients are classified

as type 1a, in which there is a direct evidence of autoimmunity shown to be responsible for the destruction of the β cells in the islets of Langerhans[2,7]. This autoimmunity, in addition to genetic and environmental factors, plays a significant role in the development of the disease[2,7]. Type 1b diabetics consist of the minority of the type 1 diabetic patients and in contrast to type 1a, autoimmunity is not the suggested mechanism of β cell destruction. Type 1b diabetes is termed idiopathic due to the episodic nature of ketoacidosis and requirement for insulin replacement therapy[2,7]. Once again, the result of type 1 DM is an absence of insulin production, resulting in a potential life-long dependence on exogenous insulin injections. For many patients, fluctuations in blood glucose levels are common, in part due to personal responsibilities, as well as an increase in severe hypoglycemic occurrences associated with exogenous insulin therapy[9,10]. Of the most difficult of these patients to treat, are those who lose their ability to feel prodromal hypoglycemic symptoms, including sweating, tremors, tachycardia, and anxiety[9]. These individuals with brittle type 1 DM have for now become the primary recipients of islet transplants, an alternative to the sometimes challenging and dangerous exogenous insulin therapies, which potentially can cause a coma, seizures, or fatality[9,10].

1.2.1 Etiology of Type 1 DM

While much has been determined about type 1 DM, a complete understanding of the etiology of the disease is still desired. Cell-mediated

autoimmune attack on the β cells of the islets of Langerhans is the main component of type 1 DM[2]. Genetic, immunologic, and environmental factors are believed to contribute in combination to the development of the disease.

1.2.1.1 Genetic Component

Genetics have been shown to play a role, but evidence has revoked full responsibility for the onset of the disease. Case in point, an identical twin is only 25-50% susceptible to the disease when the other sibling has contracted it, providing evidence that genetics alone do not predispose one to the disease[11,15]. While several genes have been linked to type 1 DM, the HLA genes found on chromosome 6, specifically HLA class II alleles or combinations of alleles, appear most strongly connected to the chances of manifesting the disease[3,11,16]. An example is the strong correlation seen between the development of diabetes and specific DQA and DQB genes, along with some DRB genes[3,16]. By understanding that HLA genes encode for MHC complexes, which are imperative for antigen presentation to $CD4^+$ and $CD8^+$ T cells, it provides some further support for their role in the disease, which is a result of dysfunctional cell-mediated immunological actions[16].

1.2.1.2 Immunological Component

Inflammation of the islets of Langerhans, or insulitis, along with the presence of circulating autoantibodies that react with islet cell

autoantigens, are the main indication of the existence of autoimmunity in type 1 DM[16]. Insulin, GAD65, as well as certain islet tyrosine phosphatases (IA2 and IA2β or ICA512) are three of the major identified autoantigens[3,11,16]. It is apparent that autoantibodies to at least one of these autoantigens will be present in approximately 80-90% of recently diagnosed type 1 DM patients[3,11,16]. Although autoantibodies themselves do not appear to cause the disease, the roughly 3.5-4% of individuals without diabetes who have the autoantibodies are at a high risk of contracting the disease at a later date[11]. This is demonstrated by the fact that adoptive transfer of the autoantibodies does not result in development of the disease, while transfer of T lymphocytes does[11].

1.2.1.3 Environmental Component

Although environmental contributions to type 1 DM are not well characterized, the gap in etiology provided by the contributions of the other two factors appears to point to some contribution from environment. Dietary factors, along with viral infections, each have been demonstrated to play a role in the development of type 1 DM. Coxsackie B4, rubella, mumps, rotavirus, and cytomegalovirus are all enteroviruses which have shown loose association with the disease[11,17]. It has been shown that dietary factors such as early exposure to cow milk may increase the likelihood of developing type 1 DM, while prolonged breast feeding may decrease the incidence of the disease[11,18].

1.2.2 Anatomy and Physiology of the Pancreas

The pancreas can be found in the epigastrium and left hypochondrium regions of the abdomen (Fig 1-1). The endocrine function of the pancreas is carried out by roughly one million clusters of islet of Langerhans, which are spread throughout the pancreas, but focused in the body and tail[1]. Primary cells in the islets of Langerhans include the β cells, α cells, δ cells, ε cells, and PP cells, which secrete insulin, glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively[1].

The essential, anabolic hormone insulin plays a role in regulating fuel mobilization and storage, promoting growth, and is a requirement for achieving metabolic homeostasis[11,12]. Postprandial insulin secretion, responsible for approximately 50% of daily insulin secretion, promotes the utilization and storage of fuels within the body. This is accomplished by suppression of hepatic glucose production (gluconeogensis and glycogenolysis), lipolysis, and proteolysis, stimulation of glycogen synthesis (glycogenesis), as well as an increased transport of glucose to myocytes and adipocytes[11,12]. Insulin is synthesized as a preprohormone in the β cells of the islets of Langerhans, and is converted to the precursor proinsulin in the rough endoplasmic reticulum of the β cells, which is cleaved to form insulin and C-peptide[12,13]. Insulin and C-peptide are later secreted in a 1:1 ratio into the hepatic circulation[12,14]. C-peptide, however, has a half-life at least twice as long as insulin's, giving a more stable concentration in the peripheral circulation[12,14]. The ability of C-peptide to

remain in the circulation longer than insulin makes it a stronger tool for reliable measurements of pancreatic insulin secretion[12,14].

In the presence of an increased concentration of glucose in the blood, ATP production in the β cells is stimulated[12,14]. Consequently, there is an inhibition of the K^+ATP channels, resulting in an influx of Ca^{2+} into the β cells, which activates the voltage gated Ca^{2+} channels[12]. The increased levels of intracellular Ca^{2+} ultimately stimulate exocytosis of insulin vesicles, releasing insulin and C-peptide into the circulation[12,14].

Glucagon counteracts the effects of insulin, stimulating the hydrolysis of glycogen by the liver, thus elevating blood glucose levels in the body. Somatostatin inhibits both insulin and glucagon secretion, while the function of pancreatic polypeptide remains undetermined[1].

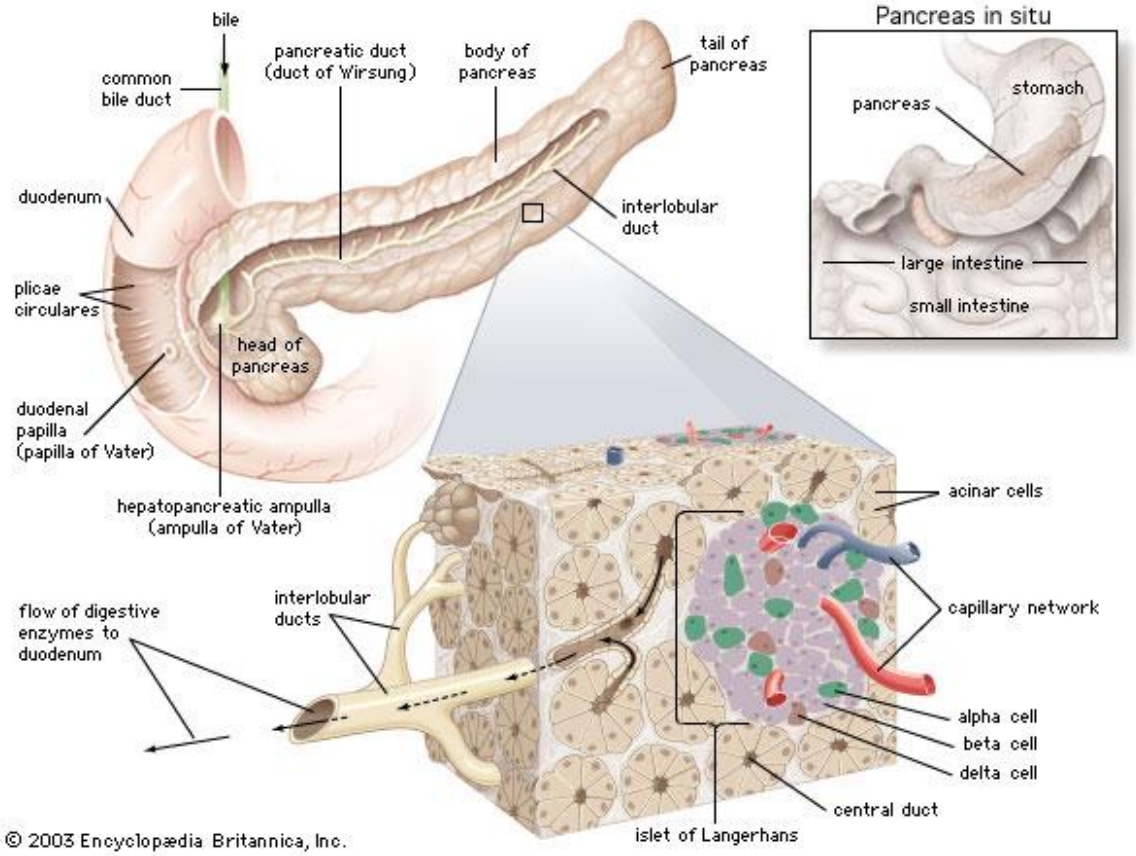

Figure 1.1: A detailed perspective of the pancreas, including an islet of Langerhans. Adapted from: Encyclopedia Britannica Online, http://www.britannica.com/EBchecked/media/68636/Structures-of-the-pancreas-Acinar-cells-produce-digestive-enzymes-which

1.2.2.1 Challenges in Metabolic Regulation in Type 1 DM

Destruction of insulin-producing β cells in individuals of type 1 DM leaves patients with an absence of insulin circulating throughout the blood[2]. As most of the body's cells require insulin to utilize the glucose from the blood, the body will continue to increase glucose production by breaking down fats and proteins, acting as if in a state of starvation[4]. Compounding the problem, continued production of glucagon and growth

hormone will lead to further endogenous glucose production[4]. Because most of this excess glucose is not taken up by the cells, the majority is excreted through urination[4].

Previously it was noted that one of the roles of insulin was inhibiting lipolysis. Therefore, in type 1 DM patients, lipolysis will not be suppressed due to the prolonged absence of insulin secretion, leading to an increase in the amount of free fatty acids. This can be quite detrimental, as increased ketogenesis eventually will cause ketoacidosis, which is a major cause of morbidity and mortality in type 1 DM patients[4]. Acidosis can be fatal as it interferes with enzymatic processes, and enhances circulatory failure, amongst other complications[4]. Similarly, the lack of insulin production prevents inhibition of proteolysis, which leads to muscle atrophy and fatigue[4].

1.2.3 Clinical Features and Diagnosis for Type 1 DM

Although it can present itself at any age, type 1 DM often affects patients before the age of 30[2,4,11]. The clinical symptoms, once again, include polyuria, polydipsia, polyphagia, weight loss, fatigue, blurred vision, and persistent hyperglycemia[1,3]. Beyond display of these symptoms, diagnosis of diabetes includes a fasting plasma glucose level (no caloric intake for duration of 8 hours prior to testing) exceeding 7.0 mmol/L, along with a random glucose level above 11.1 mmol/L[11]. A very powerful method for distinguishing type 1 DM from type 2 DM is the C-

peptide assay, a technique which measures only endogenous insulin due to the absence of C-peptide in the exogenous form of the hormone[12,14.] Another tool, which can be used to verify prolonged hyperglycemia, is elevated glycated hemoglobin A1C levels[11].

1.2.4 Treatments for Type 1 DM

Exogenous insulin is a necessity for type 1 DM patients, as insulinopenia is the dominant trait associated with the disease. For this reason, the discovery of insulin by Banting and Best in 1921 has been paramount in changing the outcome of the disease, from a previously fatal condition to a chronic one[4]. Exogenous insulin therapy continues to remain the optimum treatment for type 1 DM patients, and combined with patient education, a controlled diet, and continuous monitoring of blood glucose levels, it has been effective in lowering blood glucose levels, reversing acute complications associated with the disease, as well as improving both fat storage and muscle building[19]. The specificity of treatment and greater control of blood glucose levels have become a byproduct of the advancements in not only the types of insulin, but also the delivery systems. Blood glucose levels for bolus and maintenance requirements have been afforded more accurate regulation thanks in part to short-acting insulin analogues such as Novorapid, Humalog, and Aprida, as well as long-acting options such as Levemir and Lantus[19]. A major limitation still exists with this treatment, even with the improvements, as exogenous insulin is still inferior to functional islets in its ability to

strictly regulate a patient's blood glucose levels. A study carried out by the Diabetes Control and Complications Trial Research Group demonstrated that intensive therapy focused on strict regulation of glycemic levels significantly decreased the development and progression of secondary complications associated with type 1 DM including neuropathy, nephropathy, and retinopathy[20]. This intensive insulin therapy has its drawbacks for patients as well, increasing severe hypoglycemia by three times, potentially leading to a coma, seizures, or death[20]. The risk is further intensified for individuals with type 1 DM who already have an increased risk of hypoglycemic episodes, as well as for patients who experience poor control of glucose levels despite being treated with insulin therapy. These potential future complications thus eliminate intensive insulin therapy as a suitable treatment for some patients with type 1 DM. On the whole, while insulin therapy provides a viable option for many type 1 DM patients, in hopes of preventing future complications, future research is being chiefly focused on more physiologic solutions, including improving protocols for the transplantation of functional islet tissue.

1.3 ISLET TRANSPLANTATION

1.3.1 History of Islet Transplantation

In 1889, von Mering and Minkowski were able to establish a connection between the disease diabetes and the pancreas[21]. This linkage was discovered when polyuria and glycosuria were noted as

consequences of the surgical removal of a dog's pancreas[21]. Their discovery played a role in clinical transplantation of insulin producing tissue being practiced before insulin was known. In 1892, Minkowski attempted to reverse diabetes in diabetic dogs by subcutaneous, autologous transplantation of pancreatic fragments[22]. The following year, Watson-Williams and Harsant would attempt a procedure of similar nature clinically. They tried to treat a fifteen year old male patient with fatal ketoacidosis by transplanting whole fragments of sheep pancreas subcutaneously, having short term success in improving the boy's condition[23]. However, the lack of knowledge about xenorejection led to the eventual failure of the experiment, as the absence of immunosuppression was ultimately responsible for graft rejection, and the death of the boy three days later[23].

Improvements in islet isolation techniques allowed for the more practical transplantation of islets, as opposed to pancreatic fragments. Claus Hellerström led the charge, using free-hand microdissection to isolate rodent islets in 1964[24]. Islet yield was subsequently improved by Moskalewski the following year, who was first to combine bacterial collagenase with mechanical disruption of the pancreas[25]. Lacy and Kostianovsky were able to further improve this digestion method, and were able to isolate 300 islets by cannulating the common bile duct and distending the pancreas with Hanks media before using collagenase to mechanically and enzymatically digest rat pancreases[26]. This enabled

them to demonstrate for the first time that a sufficient amount of viable islets could be isolated for transplantation purposes. Lindall, Steffes, and Sorenson, later used differential density elutriation with Ficoll to improve islet purity, while continuing to preserve the function of the islets[27].

The improvements in islet isolation technique allowed for experimentation in islet transplantation to begin taking place in rodent models. Although only temporary success was achieved controlling blood glucose levels, Younoszai and colleagues were the first group acknowledged to successfully transplant rodent islets into a diabetic rat model in 1970[28]. In 1972, Ballinger and Lacy found that transplanting 400-600 syngeneic islets into the thigh muscle or peritoneal cavity led to significant improvement of numerous diabetic complications in streptozotocin induced diabetic rats, including sustained reversal of chemically induced diabetes never seen before[29]. They went on to demonstrate that removal of the islet isograft returned the rats to a state of hyperglycemia[29]. Furthermore, azathioprine treated diabetic rats showed a reduction in the severe diabetic state for significant periods following transplantation of islet allografts[29]. Injection of 800-1200 islets into the intraperitoneal cavity of hyperglycemic rats was shown by Reckard, Ziegler, and Barker to completely reverse the diabetic state, an achievement never accomplished previously[30]. Further development of numerous new islet isolation techniques followed as digestion of the pancreata of larger animals often produced poor yields and viability.

Refinements included intraductal infusion of Liberase, the development of a semi-automated dissociation chamber, and the utilization of a COBE cell processor for islet purification[31]. These improvements allowed for the acquisition of viable islets with increased purity and improved reproducibility, promoting large-scale isolation and strengthening the clinical applicability of the procedure[31].

Due to the morbidity and mortality linked to whole pancreas transplants, it was hoped that the improvements in islet isolation and the success in animal models would allow for islet transplantation to take over[31]. Variable results were reported for the 56 attempts at clinical islet transplantation that took place between 1977 and 1989[32]. In 1980, Largiader *et al.* reported a single instance which insulin therapy was stopped in a patient receiving a synchronous kidney-islet transplant[216]. Nine years later, Warnock and colleagues were able to demonstrate sustained C-peptide secretion after a synchronous implantation of islets into the portal vein, as well as a kidney from the same donor[32]. They then found success by increasing the marginal mass of islets transplanted by augmenting freshly isolated islets with cryopreserved islets from additional donors, citing the first patient to achieve insulin independence for an entire year post-transplantation[217]. Amongst the successes, prolonged insulin independence and reproducibility were two things that continued to evade researchers as only 8.2% of the 267 islet transplant patients in the 1990's maintained normoglycemia for one year without dependence for insulin[33].

Due to the success of prednisone, azothioprine, and cyclosporine in whole organ transplantation, a similar immunosuppressive regimen was generally utilized in islet transplantations often performed alongside kidney transplantation. Glucocorticoid based immunosuppressive regimens were then discovered to be toxic to the transplanted islets, limiting further success of islet transplantation[211,212].

In 2000, a group at the University of Alberta was able to change all of this by successfully transplanting islets to seven out of seven patients resulting in insulin independence for greater than one year post transplant[33]. They accomplished this by utilizing a mean of 11,547 islet equivalents per kilogram of recipient's body weight with a glucocorticoid-free immunosuppressive regimen consisting of sirolimus, tacrolimus, and daclizumab[33]. The study, later coined the Edmonton Protocol, was able to demonstrate that patients experienced excellent glycemic control and witnessed an end to hypoglycemic episodes following transplantation[33,34]. Furthermore, the study was able to display islet transplantation as a reproducible, minimal risk procedure capable of sustaining the freedom of insulin independence, providing a realistic treatment option for patients with type 1 DM[33,34].

1.3.2 Major Barriers to Widespread Application of Islet Transplantation

Several challenges are met with the attempt to make islet transplantation a viable, widespread mode of treatment for type 1 DM patients. Current barriers include a shortage of donor islets, selection of an optimum site for transplant, the need for continuous use of immunosuppressive drugs prevent immune mediated rejection of the islet graft, as well as the potential relapse of autoimmunity.

It is very difficult to meet the demands for pancreatic tissue needed for islet transplants into patients with diabetes, but compounding the problem, it has been shown that often patients will require a second or third transplant in order to sustain insulin independence[33,34]. A five-year follow up study to the Edmonton Protocol displayed that while 80% of patients had C-peptide present, roughly only 10% maintained insulin independence[34]. While future studies must be completed, potential sources for defects in insulin secretion may include an inadequate islet mass, increased insulin resistance in the patients, or impaired graft function potentially caused by immune mediated rejection or the deleterious effects of the immunosuppressive drugs[34]. Another stress on the shortage of donor tissue includes the potential for a lack of proper engraftment in islet transplantations. One of the leading causes commonly linked to insufficient islet engraftment is the instant blood mediated inflammatory reaction (IBMIR), a thrombotic and inflammatory

process. When islets are infused into the recipient, a clotting reaction (IBMIR) is triggered by MCP-1, a tissue factor expressed by the islets, resulting in encapsulation of the islets by a fibrin clot, along with infiltration of granulocytes and monocytes[35-37]. Antigen presentation is amplified by IBMIR, evoking an immune response directed towards the islet graft, ultimately disrupting its function. A byproduct of the clotting involved in this reaction is that access to blood vessels is prevented, further inhibiting the proper engraftment of the islet tissue[38]. Researchers have tried several methods to combat this, including the use of heparin[36], low molecular weight dextran sulfate[39], compstatin[40], and thrombin inhibitor[41]. One recently investigated option though, using genetically modified donor pigs, has added appeal due to its potential use as part of islet xenotransplantation in an effort to overcome the shortage in donor tissues[42,43].

While it appears approximately 90% of clinical islet transplantations are infusions into the hepatic portal vein, subsequently followed by embolization in the liver, it is not considered as an optimal site[44]. A number of alternative sites have been investigated, including the spleen, pancreas, kidney capsule, peritoneum, and omental pouch with varying degrees of success[44]. Although transplantation into the portal vein provides some advantages, including the fact that a relatively low amount of islet tissue is required to achieve insulin independence as opposed to other attempted transplant regions, there are still several complications

associated with it[44]. Not only is the liver site coupled with procedural complications, including severe bleeding and thrombosis, but due to the introduction of the islets into the portal circulation, the islets are subject to IBMIR, leading to consequent losses in engrafted tissue[44,45,46]. After removal from the liver, intrahepatic islets often show signs of functional irregularities that have been suggested to be attributable to the exposure in the liver to high concentrations of glucagon, diabetogenic immunosuppressive drugs, and toxins from the gastrointestinal tract[46]. Along with this, biopsies needed for the examination of the graft can be difficult and present some risk. These complications associated with infusion of islets into the hepatic portal vein create a desire to attempt to discover a safer and more reliable transplantation site for clinical islet transplantation.

In order to overcome immune mediated destruction of the islet graft, recipients are subject to continual use of toxic immunosuppressive drugs, exposing them to potentially harmful side effects and limiting the potential recipients of an islet transplant. Side effects of the immunosuppressive drugs reported in the five-year follow up of the islet transplant patients under the Edmonton Protocol include diarrhea, ovarian cysts, mouth ulcers, acne, anemia, edema, and pneumonia[34]. Due to the harmful nature of the immunosuppressive protocols on islet transplant recipients, research is currently focused on developing new anti-rejection therapies, including attempting to promote graft tolerance as opposed to targeting

immune suppression. However, preventing graft rejection becomes irrelevant if the transplanted islets are subject to the recurrence of autoimmune attack, posing another barrier to islet transplantation. It has been reported that diabetic patients indefinitely retain auto reactive T cells, indicating that there is the potential for continual autoimmune attack of islet graft tissue[47]. Recent studies show that there is promise for the potential use of regulatory T cell therapy to provide protection against autoimmunity in islet transplantation, citing the immunosuppressive properties of the secreted cytokines IL-10 and TGF-β[48].

1.4 POTENTIALSOLUTIONS TO OVERCOME MAJOR BARRIERS IN ISLET TRANSPLANTATION

1.4.1 Xenotransplantation of Neonatal Islets

While xenotransplantation appears to be a current potential aid in overcoming the shortage in donor islet tissue, it was interestingly implemented well before the discovery of insulin by Watson-Williams and Harsant in 1893, when they utilized sheep pancreatic fragments for transplantation into a boy with fatal ketoacidosis[23]. Numerous other xenogeneic sources of islet tissue have been attempted in islet transplantations including bovine islets[49-51], and fish Brockmann bodies[52-54]. Along with these, the source that has perhaps shown the most promise clinically is porcine islets, due to the high degree of similarity

physiologically and morphologically to humans[55-59]. Furthermore, the structural similarity shared amongst human and porcine insulin has been exploited clinically for years, treating patients with type 1 DM successfully with insulin from pigs[58-59]. Other benefits of using porcine tissue for xenotransplantation are the potential for rapid breeding, due to short gestation times as well as large litter sizes, along with the ability to house pigs in pathogen-free environments[56,60]. Finally, porcine islet tissue can be made safer for transplantation through genetic modification of pig donors[42].

1.4.1.1 Optimum Age for Porcine Islets for Xenotransplantation

While the use of porcine islets has been investigated by numerous groups, there has yet to be agreement on whether fetal, neonatal, or adult porcine islets will maximize the benefit of the transplanted tissue.

1.4.1.1.1 Fetal Porcine Islets

Compared to adult porcine islets, fetal porcine islets, as well as neonatal porcine islets, are considered to have greater viability and are less prone to damage at the culture and cryopreservation stages[56]. Fetal islets have the ability to be cultured for up to 30 days, allowing the islets time to mature, and thus increase insulin production[61]. Approximately 10,000 islets can be isolated from a fetal pig, which has been demonstrated to be sufficient for achieving normoglycemia in alloxan induced diabetic mice in a two month time frame post transplant[55].

Additionally, a 1994 study by a group in Uppsala, Sweden, showed that fetal porcine tissue was able to survive in the human body, transplanting ten patients with type 1 DM[63]. However, while C-peptide was secreted in the urine by four of the patients for 200-400 days, none of the patients reversed their dependence on insulin[63].

Fetal porcine islets also have many disadvantages, including the decreased number of isolated islets compared to the neonatal and adult pigs, consequently resulting in an increased number of fetal pigs necessary for a sufficient number of islet clusters. This is best demonstrated by the Uppsala group above, which required 39-100 pig fetuses in order to obtain an adequate number of islets per patient[63]. A couple major limitations of fetal porcine islets stem from their immaturity and response to glucose. Even though they demonstrate the ability to proliferate, fetal porcine islets often take months to reverse hyperglycemia in animal models, and steadily provide a relatively low secretory response towards glucose. Fetal porcine islets also contain the terminal carbohydrate epitope Galα(1,3)Gal, an antigen which appears to be a major target of the human immune system, leading to potential hyperacute rejection post transplantation[64-66].

1.4.1.1.2 Neonatal Porcine Islets

Neonatal porcine islets provide several advantages above and beyond some of the beneficial characteristics shared with fetal porcine

islets. Neonatal porcine islets are similarly easy to isolate and maintain in culture, while they provide suitable insulin secretion in response to glucose, and also consist of endocrine precursor cells that possess the potential to proliferate and differentiate into insulin producing cells following the transplant[56,60,67]. Neonatal pigs also require significantly less care time than adult pigs, reducing resources utilized to house and maintain the animals in a pathogen free environment prior to transplantation[68]. Another important advantage of neonatal pigs is the ability to isolate higher yields than from fetal pigs. Pancreatic tissue from neonatal pigs tends to be less fibrous, allowing for a more reproducible procedure, yielding typically 50,000 islet cell clusters per pig[56,67]. Although requiring up to eight weeks, it has been demonstrated that in alloxan-induced diabetic nude mice, 2,000 neonatal porcine islets were sufficient to reverse hyperglycemia[67]. In pre-clinical trials with non-human primates, Rhesus macaque monkeys have been used to demonstrate the ability of neonatal porcine islet transplants to reverse the diabetic state for prolonged periods, providing further evidence of the potential applicability of this procedure clinically in the future[69]. Although a recent clinical trial transplanting neonatal porcine islets in combination with Sertoli cells has been done in Mexico, reporting 6 of 12 patients achieving a decreased insulin dependence for as long as four years, the results have been disputed internationally, still leaving uncertainty in potential success clinically[69,70].

While neonatal porcine islets appear very appealing to future clinical success in xenotransplantation, there are some drawbacks along with the delayed *in vivo* function of the islet transplant. Like fetal porcine islets, neonatal islets also express the antigen Galα(1,3)Gal, making these transplants potentially susceptible to hyperacute rejection[71,72]. It has been demonstrated, however, that this is not likely the only xenoantigen playing a role in rejection[71]. Finally, the amount of donor pigs required and the intensive work involved in preparing these neonatal porcine islets for transplant places further limitations in their future applicability.

1.4.1.1.3 Adult Porcine Islets

Ricordi and colleagues were able to isolate 255,000 islets from an adult pig source, with an islet purity of 85-90%, utilizing a modified version of the automated human islet isolation protocol[73]. These adult porcine islets were determined to be capable of reversing hyperglycemia in diabetic nude mice[73]. The major advantages of adult porcine islets were witnessed in their experiment, including a much larger number of isolated islet clusters, reducing the number of pigs necessary for potential xenotransplantation, as well as the ability to isolate mature islet cells that increase the likelihood of optimal engraftment and provide immediate functionality upon transplantation[73,74]. Hering and colleagues were also able to demonstrate the ability of adult porcine islets to function in non-human primate pre-clinical trials, reporting that 12 cynomolgus macaques

transplanted intraportally with adult porcine islets were all able to achieve insulin independence after transplant[75].

Adult porcine islets, however, have disadvantages as well. For example, isolated adult islet clusters are very fragile, and it is very difficult to culture them in preparation for cryopreservation or in attempt to reduce immunogenicity[73,76]. The potential for increased immunogenicity may also increase the need for immunosuppressive measures, something that current research is attempting to avoid[77]. In addition, because islet yield depends on so many factors, including the breed and age of the pig, organ quality, as well as the activity and lot of the collagenase used, there can be significant variability experienced in the isolation protocol[78-80]. As mentioned previously, it would be very costly to maintain the pigs in a pathogen free environment for the required period prior to transplant, as well as very difficult to have pigs of the proper age available for transplant if long wait periods precede islet isolation[68].

While it appears that many researchers are beginning to agree that neonatal and/or adult porcine islets appear to be the best choice moving forward with clinical application, several believe that all three have strengths important to the future of clinical islet xenotransplantation[70,80].

1.4.2 Other Challenges Progressing with Clinical Xenotransplantation of Porcine Islets

1.4.2.1 Transmission of Pathogens and Viruses

One major concern when considering xenotransplantation is the prevention of xenozoonosis. Recipients of islet transplants may incur the risk of inheriting a transmissible pathogen or virus from the xenogeneic donor tissue[81]. Even worse, some of these pathogens or viruses may also have the ability to be transmitted across human cell lines, putting further human population at risk[81]. Although attempts can be made to ensure breeding occurs in pathogen free environments, some endogenous viruses, including the Porcine Endogenous Retrovirus (PERV), can be very difficult to screen out[82]. Even though xenotransplantation of porcine tissue has not appeared to yield any cases of long-term infections of transplant recipients with PERV, including patients under immunosuppressive regimens, the ability of PERV to infect human kidney cell lines *in vitro* demonstrates the potential risk involved[67,83-85].

1.4.2.2 Xenograft Rejection of Porcine Islets

Another major challenge moving forward with xenotransplantation of porcine tissue is the immune response mounted against xenogeneic tissue. In order to increase clinical applicability, obtaining methods to evade the potent immune response aside from immunosuppressive therapies is of the utmost importance. First, the mechanisms by which

xenografts are rejected must be understood. Innate, antibody, and cellular mediated immune responses, along with IBMIR, each play a role in rejection of xenogeneic tissue.

Instant blood-mediated inflammatory reaction has the ability to destroy functionality of the islet graft immediately after coming into contact with host blood without the assistance of the adaptive immune response, potentially destroying up to 73% of the transplanted xenogeneic tissue within the first day post transplant[86]. Thus, in order to achieve prolonged insulin independence, there must be a significant increase in the number of transplanted islets[69]. The presence of the Galα(1,3)Gal epitope on porcine tissue appears to amplify the effects of IBMIR when dealing with xeno- as opposed to allotransplantation, due to pre-formed antibodies and complement towards this common mammalian antigen[71-72]. The effects of IBMIR have also been shown to include other immunological pathways aside from the complement component, as IBMIR was still a factor in xenograft destruction, albeit reduced, under complement inhibition by cobra venom factor[87]. The IBMIR presents an ongoing challenge striving for clinical application of porcine islet xenotransplantation.

The innate immune response is also apparent in porcine islet xenograft rejection and it has been determined that this innate immune response is governed by natural killer cells, macrophages, and eosinophils[88-90]. While it seems they all have their importance in xenograft rejection, studies appear to show that specifically the depletion of

macrophages has the strongest relationship with prolonged graft survival, and that macrophages are capable of effectively causing rejection by damaging xenogeneic tissue[90-92].

Transplanted tissue is subject to two different pathways of T cell mediated rejection. In the direct pathway, host T cells recognize antigens apparent on the surface of donor antigen presenting cells (APC)[93]. In the indirect pathway, host T cells recognize antigens, which are processed and presented by host APC[93]. Along with this interaction between T cell and the antigen, a second co-stimulatory signal provides further amplification, stimulating activation of the T cell. Both pathways are capable of undergoing T cell activation, leading to an immune response capable of being responsible for the destruction of transplanted tissue. While allograft rejection is dominantly subject to the direct pathway, Rayat and Gill showed that the importance or role of the indirect pathway of antigen recognition increased as the phylogenetic disparity between host and donor increased[94]. Differing evolutionary relationships thus have an effect on the type of T cell mediated response effectively responsible for rejection of the xenograft. Targeting the indirect pathway in discordant porcine islet transplants into recipient mice has been shown to be effective at reducing the rejection of xenograft tissue[94]. While knowledge of the importance of $CD4^+$ T lymphocytes and the indirect antigen recognition pathway in a discordant relationship between donor and recipient may not translate ideally when interchanging species, understanding the

mechanisms behind xenograft rejection and targeting such potential systems is important in moving forward with the clinical application of porcine islet xenotransplantation in type 1 DM patients. Furthermore, discovering methods to induce tolerance in transplanted xenogeneic tissue will significantly reduce complications associated with long-term use of immunosuppressive drugs.

1.4.2.3 Preventing Recurring Autoimmune Attack on Islet Xenograft Tissue

Due to the fact the main recipients of islet transplants are patients with a previous autoimmune disorder, a major concern looming with xenotransplantation is whether these patients will have a recurring autoimmune attack towards the new islet xenograft[96,97]. Studies appear to demonstrate some partial species specificity in regards to autoimmune response[96,97]. While the extent of specificity is undetermined, it has been demonstrated that if autoimmune cells and antibodies are capable of targeting xenogeneic tissue, it may be destroyed by the host immune system[94].

Studies have shown that strategies effective in preventing xenograft rejection in chemically induced diabetic mice cannot be similarly efficacious in a spontaneously diabetic non-obese diabetic mouse autoimmune model[214]. The short-term administration of anti-LFA-1 and anti-CD154 monoclonal antibodies was a highly effective strategy in

inducing tolerance to neonatal porcine islet xenografts in B6 mice, but failed to promote similar xenograft protection in NOD mice[214]. However, subsequent addition of a $CD4^+$ T cell depleting monoclonal antibody promoted xenograft survival in nine of 12 NOD mice, including long-term graft survival of greater than 100 days in 2 mice[214]. It has also been shown that adult porcine islet xenograft survival could be prolonged in NOD mice by a $CD4^+$ T cell depleting approach[215]. It was also demonstrated that efficacy was not improved by further depletion of $CD8^+$ T cells. Supplementary studies, which treated NOD mice with streptozotocin to chemically induce diabetes and bypass the autoimmune response prevented rejection of islet isografts in NOD mice. $CD4^+$ T cell depletion yielded similar results in graft survival of adult porcine islets transplanted in streptozotocin treated and spontaneously diabetic NOD mice[215]. While these results appear to indicate that recurrent autoimmunity may not be a major contributor to xenograft rejection, it must be noted that autoreactive $CD4^+$ T cells may be suppressed by the monoclonal antibody therapies, and that the inability to achieve indefinite graft survival may be due to recuperation of the $CD4^+$ T cell population[215].

1.5 STRATEGIES IN PREVENTING THE REJECTION OF PORCINE ISLETS

1.5.1 Monoclonal Antibodies: Anti-LFA-1 Monoclonal Antibody Therapy

As mentioned previously, two signals are necessary and important for T cell activation, including antigen recognition by the T cell, and the secondary co-stimulatory interaction. The activation of T cells leads to a production of cytokines, as well as proliferation and differentiation of effector cells, causing an immune reaction[98,99]. This makes the T cell activation pathway, along with co-stimulatory molecules excellent targets for monoclonal antibody therapy, in attempt to prevent an immune response ultimately causing xenograft rejection[100].

Leukocyte function associated antigen-1 (LFA-1) is an important adhesion molecule expressed on a number of different hematopoietic cells including macrophages, monocytes, granulocytes, natural killer cells, and T and B lymphocytes[101-103]. Primary ligands of LFA-1 are ICAM-1 and ICAM-2 on endothelial cells, ICAM-1 and ICAM-3 on APC, and JAM-1 which is present on tight junctions of epithelial and endothelial cells[104,105]. This adhesion molecule has several roles, which may provide a beneficial target for a therapy combating immune rejection of xenograft tissue.

LFA-1 plays the role of an integrin, responsible for binding to ICAM-1 in order to arrest rolling leukocytes on the endothelial wall. As activated

T cells migrate along the endothelial surface, the firm interaction between LFA-1 and ICAM-1 prevents further rolling, allowing the leukocytes to migrate through the endothelial wall to the site of inflammation[106]. Antibodies to LFA-1 subsequently prevent this interaction with ICAM-1, preventing the leukocytes from arresting their movement[106].

Another role of LFA-1 is that it seems to provide additional signals, which lowers T cell activation thresholds. It also appears to contribute functionally to effector cell differentiation, polarizing T cells towards T_H1[107]. When LFA-1 interacts with ICAM-2, phosphorylation of the β2 chain of LFA-1 occurs, resulting in c-Jun phosphorylation and stimulation of interleukin-2 production[107-109]. Antibodies to LFA-1 thus block the interaction between LFA-1 and ICAM-2, interfering with the phosphorylation of LFA-1, and preventing co-stimulation. By decreasing possible LFA-1 mediated co-stimulation signals, anti-LFA-1 monoclonal antibody has the ability to potentially prevent the activation of T cells[110].

The role of LFA-1 at the boundary between APC and T cells has also been researched as a potential target for therapy preventing graft rejection. While the T cell receptor binds to a specific antigen presenting MHC molecule, in order for T cells to be sufficiently activated to carry out further functions, prolonged periods of engagement are essential. These lengthy interactions are not achieved strictly through the binding of T cell receptors to MHC molecules due to steric hindrance, low affinity between T cell receptors and MHC molecules, and low numbers of antigen

complexes available for binding[111]. Consequently, adhesion molecules are necessary in addition to stabilize and prolong the interaction[111]. Abraham and colleagues demonstrated this when they found that a 10,000 fold increase in T cell receptor ligand from what would cause proliferation in the presence of the interaction between ICAM-1 and LFA-1, would still not cause proliferation in the absence of the interaction, confirming the importance of this adhesion molecule in T cell activation[112]. A recent discovery suggests that the interaction of ICAM-1 with LFA-1 in combination with T cell receptor signaling augments Ras activation, a component important in regulating T cell development, proliferation, and homeostasis[113]. These interactions involving LFA-1 all appear to produce a decrease in the T cell activation threshold, which can be a useful target for attempting to find a beneficial therapy to prevent xenograft rejection[114].

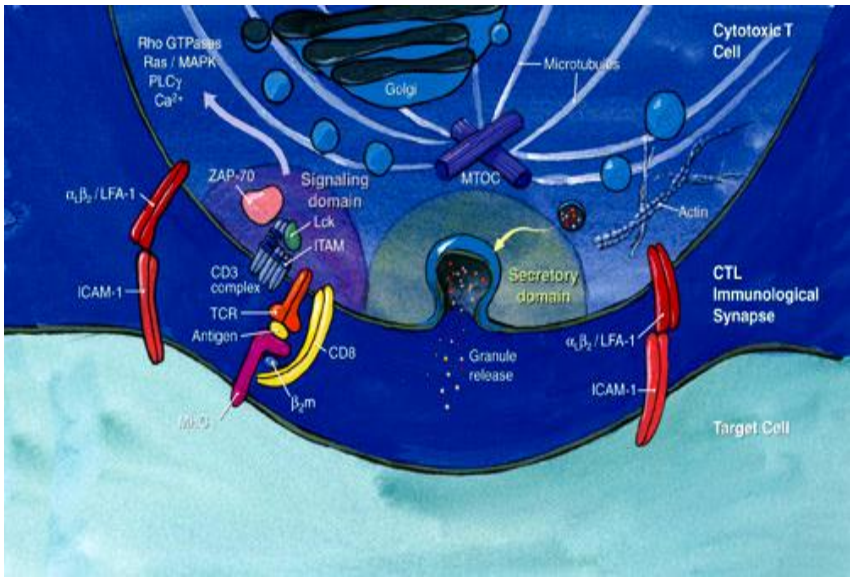

Figure 1.2: The interaction between LFA-1 and ICAM-1 aids in T cell activation. Image courtesy of R&D Systems: http://www.rndsystems.com/mini_review_detail_objectname_MR05_CytolyticGranule.aspx

Several studies have shown that antibodies targeting LFA-1 have the ability to inhibit T cell mediated killing through a number of different mechanisms, ultimately providing protection for the transplanted tissue, improving graft survival[101,115]. Mixed lymphocyte reactions, which are *in vitro* proliferation assays, have demonstrated the ability of anti-LFA-1 monoclonal antibody to inhibit the proliferation of lymphocytes towards allo- as well as xenoantigens[114,115]. Anti-LFA-1 monoclonal antibody therapy has also been shown to be efficacious in several transplant models including tracheal and cardiac models[115-119]. The ability of anti-LFA-1 monoclonal antibody to provide protection to islet allografts appears to be highly dependent on the strain of recipient mouse, as 89% of CBA

mice receiving BALB/c islets achieved long term allograft survival (>100 days), while only 39% of C57BL/6 mice receiving BALB/c islets had the same success[118]. Another interesting point in the study was that anti-LFA-1 monoclonal antibody induced allograft acceptance was able to be adoptively transferred into immune deficient mice which had received islet allografts. This demonstrated the ability of anti-LFA-1 monoclonal antibody to induce donor-specific tolerance[118]. Immunization of donor-type B6 spleen cells into BALB/c recipients with B6 islet grafts was performed in order to remove doubt that tolerance was the basis for protection, and not immunologic ignorance[118]. Anti-donor proliferative responses, cytotoxic reactivity, and cytokine release were also assessed *in vitro*, confirming that anti-donor reactivity was present in tolerant immune cells, and determining that the mechanism of anti-LFA-1 monoclonal antibody tolerance induction was not by clonal deletion or anergy[118]. Studies have shown that in allotransplantation models, anti-LFA-1 monoclonal antibody therapy alone is inadequate to sustain islet graft survival in autoimmune non-obese diabetic mice[114]. In combination with anti-CD154 monoclonal antibody therapy, however, anti-LFA-1 monoclonal antibody is able to provide a significant increase in allograft survival[114]. Prevention and reversal of diabetes has remarkably been demonstrated by targeting the interaction between LFA-1 and ICAM-1 in both autoimmune and chemically induced diabetic models[120-122].

Anti-LFA-1 monoclonal antibody therapy has also been shown to be effective in both concordant and discordant islet xenograft models. Tredget and colleagues showed that in a rat to mouse islet transplant model, short-term administration of anti-LFA-1 monoclonal antibody therapy was sufficient to prevent islet xenograft rejection for greater than 100 days in 27 of the 28 rat recipients[115]. In a contrasting discordant xenograft model, where neonatal porcine islets were transplanted into C57BL/6 diabetic mouse recipients, only seven of the 15 mice achieved sustained normoglycemia, while only six of these seven prevented xenograft rejection for longer than 100 days[119]. The combination of anti-LFA-1 monoclonal antibody with anti-CD154 extended this protection however, as 12 out of 14 mouse recipients of neonatal porcine islets achieved long-term xenograft survival[119]. Furthermore, it was later demonstrated that the combination of these two monoclonal antibodies was inducing tolerance to neonatal porcine xenografts by a T regulatory cell mediated mechanism[213]. For porcine to mouse discordant model, it appears necessary that complementary therapies are found to enhance the benefits experienced with anti-LFA-1 monoclonal antibody therapy.

Efalizumab is a humanized version of anti-LFA-1 monoclonal antibody therapy currently tried on humans which targets the CD11a α chain and has provided somewhat efficacious results for the treatment of psoriasis, although dependable effectiveness is still something that is strived for[110]. Treatment with Efalizumab has led to some acute side

effects including headache, chills, fever, nausea, and myalgia[123]. Higher doses have even been demonstrated to increase the likelihood of developing lymphoproliferative disease[124]. While anti-LFA-1 monoclonal antibody therapy appears to be potentially beneficial to xenotransplantation, more work needs to be done determining effective doses, and the potential side effects still present additional complications to patients. Even if Efalizumab is not deemed clinically safe, deeper understanding of the mechanisms responsible for the benefits of anti-LFA-1 monoclonal antibody therapy could provide insight on potential new targets focused on preventing xenograft rejection.

1.5.2 Immunological Protection and Co-transplantation with Sertoli Cells

Several locations in the human body have been coined with the term immune privileged, providing local tissues with a degree of protection against the immune system. Consequently, it has been demonstrated that transplanting in these immune privileged sites has had a positive effect on prolonging graft survival[125]. Such determined immune privileged locations include the brain, placenta, anterior chamber of the eye, as well as the testes[126]. After understanding the significance of potential damage to some of these regions, including impaired capacity to function or reproduce, it can be seen how this immune privileged feature would be advantageous to retain as generations accumulate. The testis have become an area of great interest due to an ability to prevent immune

rejection of post-meiotic germ cells capable of evoking an immune response because of their unique surface antigens, along with being able to prevent inflammation reactions which would encourage bystander killing[127]. Although it was previously believed that segregation from the immune system was responsible for this immune protection, new information has changed thoughts, indicating that ignorance on the part of the immune system is not the only factor involved in enabling the testis to maintain an environment providing protection from attack by host immunity[127,128]. Contributing evidence to this claim includes regions such as the rete testis, where the blood-testis barrier experiences incomplete permeability, providing an opportunity for T cells to enter and for soluble sperm cells to exit. Furthermore, despite lymphatic drainage commonly taking place in the testis, they are still able to provide protection to autoantigenic tissue[127,128]. While much debate still exists over immune privilege, the ability of the testis to provide a degree of protection against the host immune system is an intriguing thought for islet transplantation.

A subsequent method of transplantation has evolved from these findings, co-transplanting islets with Sertoli cells in an effort to provide the islet graft with immunological protection and trophic support[129,130]. Enrico Sertoli was responsible for the discovery of Sertoli cells in 1865[131]. Sertoli cells are found in the testis, and make up a section of the seminiferous tubules. Along with being responsible for forming the blood-testis barrier through tight junctions, Sertoli cells also nurture developing germ cells,

secrete numerous hormones and growth factors, and play a role in preventing germ cells from being attacked by the immune system[131,132]. Sertoli cells are known to provide an immunosuppressed environment somewhat due to the nature of secreted products, which have a deterring effect on the inflammatory response. These products have demonstrated an ability to reduce proliferation of lymphocytes as well as decrease the production of IL-2 *in vitro*, arresting cells in the G1 phase of the cell cycle[133]. Sertoli cell-conditioned media is also able to prevent exogenous IL-2 from stimulating the proliferation of lymphocytes, while also significantly reducing cytotoxic T cell mediated killing and DNA fragmentation of target cells by inhibition of granzyme B[134,135]. Studies have also shown the ability of Sertoli cells to secrete products which inhibit cell destruction via the complement system and prevent assembling of the membrane attack complex, demonstrating a capability *in vitro* to avoid human antibody-mediated lysis[136]. Four products of Sertoli cells which are thought of to be responsible for their immunosuppressive properties include clusterin, TGF-β, FasL, and serine protease inhibitors[135,137,138]. Research performed by Ramji and colleagues found that 7 out of 7 C57BL/6 diabetic mice achieved prolonged normoglycemia (>100 days) when neonatal porcine islets were co-transplanted with Sertoli cells and treated with anti-LFA-1 monoclonal antibody therapy, indicating that this combination therapy was capable of preventing immune mediated graft destruction[139]. Further research elucidating the mechanism for this

protection granted to the islet graft by co-transplantation of islets with Sertoli cells will allow for subsequent movement towards clinical applicability of xenotransplantation, potentially stimulating new research focused on these developments.

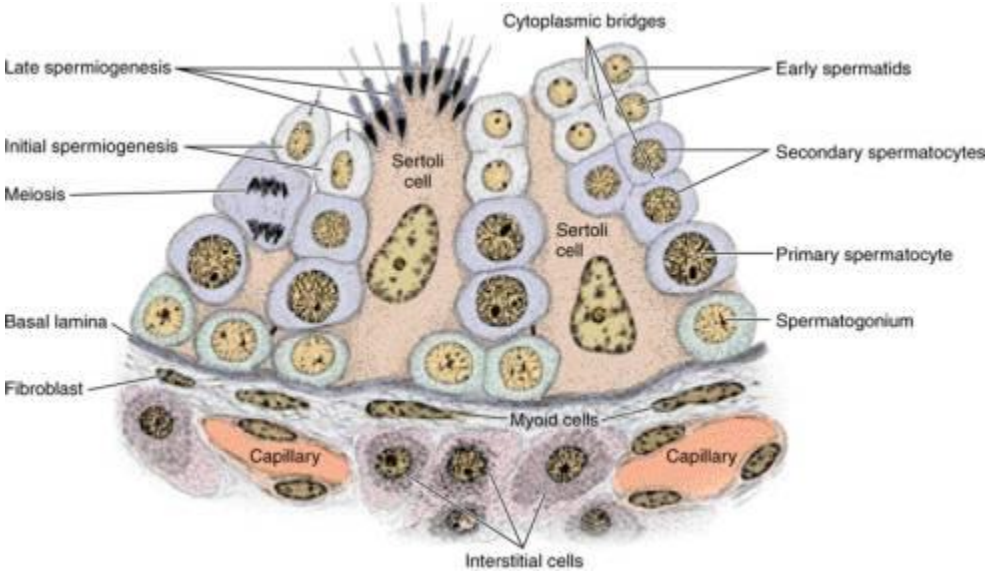

Figure 1.3: Depiction of Sertoli cells within the seminiferous tubule. Adapted from: Vanderbilt University Medical Center, http://www.mc.vanderbilt.edu/histology/labmanual2002/labsection3/TestesandSperm03.htm

1.5.3 Immune Mediated Tolerance

Immune mediated tolerance is witnessed when the immune system has no response towards an antigen in question. While the immune response still engages the majority of antigens, tolerance is defined as unresponsiveness to specific antigens associated with the donor graft when considering tissue transplantation[140]. The ability of the immune system to continue to evoke responses towards natural antigens is an

empowering trait of tolerance, providing a significant advantage over the harsh nonspecific immunosuppressive regimens which are used to prevent graft rejection in a clinical setting and are associated with several complications including increased risk of infection. Billingham and colleagues are the first group credited with reporting about inducing immunological tolerance in mouse models more than 50 years ago, and since there has been continued intense research on the topic[141]. While considerable research has focused on inducing tolerance experimentally in small and large animal models, there remains several hurdles in applying these findings into humans. However, many things that have hindered the widespread clinical application of islet transplantation, including the use of immunosuppressive drugs, chronic rejection of the islet graft, as well as a shortage of donor tissue, can be potentially solved by understanding principles of immune mediated tolerance and applying them to transplantation of xenograft tissue. Tolerance may be realized through two different mechanisms known as central tolerance and peripheral tolerance.

1.5.3.1 Central Tolerance

Central tolerance is tolerance induced in the thymus during lymphocyte development. Essentially, lymphocytes that respond to self-MHC molecules are physically or functionally removed following their development, inducing self-tolerance in the process of shaping the T cell repertoire. These lymphocytes undergoing development and maturation

in the thymus undergo positive and negative selection, with a significant proportion of cells not making it through the selection process[142]. Negative selection is especially important, as this is where T cells in the thymus which have high avidity towards self-MHC molecules are removed from the repertoire, preventing further autoreactivity in the periphery. The autoimmune regulator transcription factor *Aire* is responsible for providing a source of peripheral tissue specific antigen in the thymus, and although studies on *Aire* have favored central tolerance as the only necessary mechanism for tolerance induction, they have not ignored the potential for the peripheral mechanism playing a role[143-146]. Expression of peripheral antigens by heterogeneous medullary thymic epithelial cells is restricted to not only scattered single cells, but also to the number of antigens these cells are capable of presenting[147]. It has also been demonstrated that other transcription factors aid *Aire* in presenting these tissue restricted antigens from the periphery[148]. Although it is still unknown, in order for this to be the exclusive mechanism in inducing tolerance, transcription factors controlling thymic expression of peripheral tissue specific antigens must be able to regulate the expression of all antigens associated with the transplant, allowing T cells to recognize these antigens during development and be negatively selected for in the thymus. Generation of self-tolerance in T cells therefore is occurring in the thymus during lymphocyte development, as well as potentially in the periphery post development.

1.5.3.2 Peripheral Tolerance

Peripheral tolerance is tolerance induced after lymphocytes have developed and matured in the thymus and subsequently entered the periphery. Acceptance of allogeneic grafts of peripheral tissue in some transplantation studies, as previously talked about, has provided the support for peripheral tolerance. It is imperative to note though, that a mechanism for this tolerance induction to a peripheral tissue specific antigen has yet to be elucidated. Once again it should be stated that developing an understanding of this peripheral self-tolerance mechanism has potential to provide a new therapy strong enough to open the doors to widespread clinical application of islet xenotransplantation. Because $CD4^+$ T cells play an important role governing the reactivity of B cells and $CD8^+$ T cells, it will be important to understand the mechanisms behind $CD4^+$ mediated tolerance in order to understand tolerance as a whole[148-151]. An interesting challenge is encountered when considering peripheral tolerance in $CD4^+$ T cells, as under normal conditions, $CD4^+$ T cells only recognize antigens presented on a few cell types that express MHC class II, and the antigens they recognize are peptides which are the product of the endocytic pathway instead of proteins manufactured in the cell. There are numerous models attempting to provide general rules for deciphering peripheral $CD4^+$ tolerance from immunity, along with the specific mechanisms of tolerance involved.

1.5.3.3 Mechanisms of T-cell Mediated Tolerance

There are four major mechanisms of T-cell mediated tolerance which are recognized, including clonal deletion, anergy, ignorance, and suppression or regulation. Each of these methods may act individually or in combination with others to achieve tolerance.

1.5.3.3.1 Clonal Deletion

In the context of transplantation, clonal deletion would infer an elimination of T cells which have receptors that are capable of recognizing antigens expressed by donor tissue. Studies dictate that clonal deletion appears to be the major mechanism of inducing self-tolerance in the thymus during lymphocyte development[152-154]. Due to their ability to escape clonal deletion, T cell receptors with lower avidity for donor antigen complexes require alternate mechanisms to achieve tolerance, especially during periods of inflammation and antigen upregulation[155-157]. It has also been demonstrated that under certain conditions, mature T lymphocytes can be deleted in the periphery. For example, clonal deletion can occur as a result of mature T cells being exposed to antigen in the peripheral lymphoid tissues[158]. Secondly, under non-inflammatory conditions, dendritic cells of the lymph node can cross-present self-antigens leading to deletion of tissue antigen-specific cytotoxic $CD8^+$ T cells[159]. Furthermore, the potential for deletion of $CD8^+$ T cells exists by exhaustion of the $CD8^+$ T cell receptors by a large presence of antigen[160]. Recent

studies have also gone on to show that $CD4^-CD8^-$ regulatory T cells are capable of deleting $CD8^+$ alloreactive T cells with the same specificity as the regulatory cells[161].

1.5.3.3.2 Anergy

Anergy refers to the inability of the immune system to respond to an antigen. More specifically, it is defined as an induction of tolerance due to an inability of T cells to proliferate and produce IL-2 in response to a recognized antigen, which is typically a self-antigen. Anergy can develop when T cells receive the first necessary signal through an interaction with the MHC complex, but lack an adequate co-stimulatory second signal which is needed for a response[162]. Another situation which may be deemed anergy is when T cells interact with peptide ligands that they have a low affinity for, preventing an immune response[153]. Depending on the level and process of maturity, some antigen presenting cells such as macrophages and dendritic cells have the ability to induce anergy in T cells, somewhat in response to secretion of cytokines and an inadequate co-stimulatory signal[163-164]. Anergy is typically associated with T cell receptor down-modulation, as well as altered signaling and tyrosine phosphorylation patterns, and it can be often overcome by the addition of exogenous IL-2[162,165-169]. Because of the reversible state anergy finds itself under conditions favoring inflammation, other mechanisms must be relied on to provide support in order to maintain tolerance[170-171]. It has been demonstrated in some studies that in the presence of continual

antigen, the induction of anergy in T cells is followed by clonal deletion[172-173]. Research has also demonstrated that T cells in a state of anergy have the potential to suppress activity of other T cells, serving the function of regulatory T cells. It appears these anergic cells may condition antigen presenting cells such as dendritic cells to tolerize T cells that have the same or different specificity, thus preventing the dendritic cells from presenting antigens[174]. Furthermore, regulatory T cells share biochemical characteristics with anergic T cells, properties which potentially play a role in the ability of these regulatory T cells to induce anergy in other T cells[175-176].

1.5.3.3.3 Ignorance

Ignorance is a mechanism of tolerance in which T cell or B cell receptors recognize an antigen, but simply ignore it, consequently avoiding an immune response[169,177]. A potential cause of ignorance is the inability of the T cell to be activated due to an inadequate interaction with non-professional antigen presenting cells, which do not constitutively express MHC proteins required for this interaction[178]. Furthermore, it has been demonstrated in murine solid tumor models that ignorance may also be due to a failure of the T cells to migrate to the tissue expressing the antigen[178]. There are numerous factors which appear to contribute to the state of the T cell, such as how much time has passed since the T cell left the thymus and entered the periphery, the degree of antigen expression, and whether co-stimulatory molecules or proinflammatory cytokines are

present or absent in the tissues of the periphery[169,179-180]. Studies have shown that tolerance, in the form of ignorance, appears to be a state of uneasiness, overturned by immunological responses due to inflammation caused by infection, as well as antigen presentation by professional antigen presenting cells[140,181-182].

1.5.3.3.4 Suppression of T-cell Response

Suppression of the T cell response, sometimes referred to as regulation, consists of a cell population actively decreasing the reactivity of T cells. This is the final major mechanism of inducing self-tolerance, and has been found a contributor to tolerance in recent rodent transplantation tolerance models. Several mechanisms are known to down regulate an immune response once it has begun, and it is the net outcome between the combination of activating and suppressing actions that determines the nature of the immune response. Such mechanisms responsible for suppression of the immune response include the cytotoxic killing of antigen presenting cells, the inhibitory effects of cytokines, as well as activation-induced death. While research as far back as the 1970's implicated the potential for T cells to be actively responsible for suppression of the immune response, it was not until recently that identified molecular markers of regulatory T cells have allowed for suppressive T cell populations to be isolated, cultured *in vitro*, and adoptively transferred[140,152,155,183-184].

Numerous transplantation models have demonstrated the importance of regulatory T cells in generating and maintaining tolerance, while different protocols have successfully induced tolerance to islet allografts with simultaneous transplantation of $CD4^+CD25^+$ regulatory T cells[185-189]. Several studies have strongly indicated that suppressive $CD4^+CD25^+$ regulatory T cells are important for induction and maintenance of self-tolerance. Hall and colleagues were the first to report $CD4^+CD25^+$ regulatory T cells as a suppressive population of cells in rat recipients of cardiac allografts, and since regulatory T cells have been implicated in reported cases of accepted allografts in rodents receiving an initial, short term immunosuppressive regimen[190-196]. It has been demonstrated in xenogeneic models that $CD4^+CD25^+$ regulatory T cells appear responsible for suppressed IL-2 and inflammatory cytokines, along with a reduced cytotoxic T cell response *in vitro* towards porcine tissue[197]. Further research has indicated that these regulatory T cells develop in the thymus, require specific positive selection, and express the transcription factor Forkhead box P3, also known as FoxP3, which is responsible for regulating development and function of these suppressive cells[155,198-199]. It appears that cell-to-cell contact is required for *in vitro* suppression by $CD4^+CD25^+$ regulatory T cells, while transforming growth factor-β (TGF-β) plays a large role maintaining the regulatory cells along with regulating their suppressive activity[200-203]. Regulatory T cells have the ability to not only suppress $CD4^+$ and $CD8^+$ T cells, but also have the ability to

suppress both naïve and memory immune reactions. While it appears a specific antigen is a necessity to elicit a regulatory T cell response, the suppressive effector response is not governed by the specificity of the antigen[191,204-205]. Interestingly, IL-2 is not produced by regulatory T cells, but plays a large role in their generation, expansion, and survival, as well as playing a potential role in their function[206]. Along with maintaining peripheral regulatory T cell populations, TGF-β also can suppress T cell activation through mechanisms independent of regulatory T cells[203,207-208]. It also promotes adaptive regulatory T cell differentiation, and has the ability to regulate dendritic cell function, making them tolerogenic for T cells[163,207,209]. While these regulatory T cells have been demonstrated to be important in generating allograft tolerance in experimental scenarios, it can also be said that evidence points to these suppressive T cells playing a prominent role in maintaining self-tolerance in humans. People experiencing congenital defects in FoxP3 are often linked to autoimmune disorder, immune dysregulation, enteropathy, and polyendocrinopathy[210]. These experimental findings have peaked the interest of researchers, and work is being done to describe additional types of regulatory T cells, as well as to understand the role these suppressive populations play in mediating tolerance in numerous experimental models. With more information, and a better understanding, regulatory T cells have the potential to provide researchers with an additional tool for generating and maintaining tolerance in the clinical application of tissue transplantation.

1.6 OBJECTIVES AND OUTLINE

Recent data has reported that the combination therapy of anti-LFA-1 monoclonal antibody with Sertoli cells has been efficacious in preventing porcine islet xenograft rejection. This has focused the primary objective of this thesis on determining whether this anti-rejection protocol induces tolerance to porcine islets, as well as further elucidation of the mechanisms of proposed tolerance.

The objective of this study on the whole, is to attempt to address two of the major challenges currently facing the clinical application of islet transplantation. First, in order to overcome the potential shortage of donor tissue faced once widespread application of islet transplantation occurs, the use of neonatal porcine islets have been proposed as an alternative source of transplantable islets. Thus, in our study, we are focused on elucidating the mechanism of tolerance induced towards neonatal porcine islets, in order to develop an understanding that can be potentially transferred to human clinical application. Second, by trying to understand how tolerance is induced in our experimental model, we are attempting to remove the requirement for chronic immunosuppression associated with current islet transplantation protocols. Due to the fact these harmful immunosuppressive therapies have severely limited the widespread clinical application of islet transplantation, understanding the mechanism of induced tolerance, and potentially applying it clinically would vastly increase the number of potential recipients for islet transplantations.

Hence, the overall objective of the study is to develop and understand the mechanism of tolerance to xenogeneic islet grafts, in attempt to potentially extrapolate and exploit the knowledge in order to facilitate the widespread application of islet transplantation.

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CHAPTER 2

PROTECTION OF NEONATAL PORCINE ISLETS CO-TRANSPLANTED WITH SERTOLI CELLS COMBINED WITH ANTI-LFA-1 MONOCLONAL ANTIBODY THERAPY APPEARS TO BE MEDIATED BY REGULATORY T CELLS AND SECRETED SERTOLI CELL PRODUCTS*

2.1 INTRODUCTION

Islet transplantation has the potential to become an alternative method of treatment for individuals with type 1 diabetes, ever since the success of the Edmonton Protocol in 2000[1]. While individuals with type 1 diabetes were commonly subject to numerous daily insulin injections in order to maintain normoglycemia[2,3,4], islet transplantation has offered a more physiological approach to insulin delivery, minimizing the risk in incurring severe secondary complications[1,5-7]. There are, however, some major barriers preventing the widespread application of islet transplantation beyond patients with "brittle" type 1 diabetes[1,5,8]. One of the major barriers includes a shortage of human donor pancreases, in

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which xenotransplantation of neonatal porcine islets (NPI) has been proposed as an alternative tissue source for islet transplantation in a clinical setting. NPI are easily isolated and maintained in culture, they have demonstrated an ability to proliferate and differentiate[9-12], and they are capable of reversing diabetes in both small and large animal models[13-17], including non-human primates[18]. The second major challenge which needs to be overcome to make islet xenotransplantation potentially clinically applicable is finding an alternative to the need for continuous use of harmful immunosuppressive drugs[1,5-8]. It is imperative to find short-term therapies which can prevent initial graft rejection, and ideally promote immune tolerance. Administration of monoclonal antibody (mAb), such as anti-LFA-1 mAb, has shown to have potential to satisfy these requirements, however, mAb therapy has only proven to be moderately effective in preventing NPI xenograft rejection when used as a monotherapy[16].

One potential strategy to help prevent NPI xenograft rejection in combination with mAb therapy includes co-transplantation with neonatal porcine Sertoli cells (SC). SC have been shown to suppress proliferation of both T cells and B cells, while also displaying the ability to significantly decrease the production of interleukin-2 (IL-2) *in vitro*[19]. Numerous secreted SC products have been implicated in sharing responsibility for the anti-inflammatory and immunosuppressive properties of these cells, including clusterin, FasL, TGF-β, and serine proeinase inhibitors such as

serpina3n[20-24]. It has been shown in an NPI xenograft model that 7/7 B6 mice co-transplanted with NPI and SC and treated with anti-LFA-1 mAb successfully achieved long-term normoglycemia[17]. Notably, it has also been shown that of 8 B6 mice that were transplanted with NPI and SC with no additional mAb treatment, none were successful at achieving long-term normoglycemia[17]. This indicates that while SC are protective, they are unable to prevent NPI xenograft rejection alone, as a mAb therapy is required in addition to prevent the initial rejection of the NPI and SC xenograft. The main objective of this study is directed at determining the mechanisms behind the long-term xenograft protection of co-transplanted NPI and SC treated with a short-course of anti-LFA-1 mAb therapy.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Two to three day old female or male neonatal pigs (1.2-2.2 kg, University of Alberta farm, Alberta, Canada) were utilized for islet and SC donors. Six to eight week old male C57BL/6 (B6, H-2^b, The Jackson Laboratory, Bar Harbor, ME, USA) mice were used as transplant recipients, while six to eight week old male B6 *rag-/-* (B6.129S7-Rag1^{tm1mom}/J, H-2^b, The Jackson Laboratory, Bar Harbor, ME, USA) mice served as controls. The mice were rendered chemically diabetic by the delivery of streptozotocin (Sigma, St. Louis, MO, USA) 3-5 days prior to transplantation through a single intraperitoneal injection at a dose of 200

mg/kg and 185 mg/kg body weight, respectively. Blood glucose levels were monitored biweekly using a One Touch Ultra glucose meter (Lifescan Inc., Milpitas, CA, USA). Diabetic mice were subject to two consecutive blood glucose readings greater than 17 mmol/L prior to transplantation. All animals were given standard laboratory food and were cared for in accordance to established guidelines of the Animal Care Use Committee at the University of Alberta, along with the Canadian Council on Animal Care.

2.2.2 Isolation of NPI

Neonatal pigs were anesthetized with isoflurane, followed by subsequent laparotomy and exsanguination. The pancreas was removed by dissection and placed in Hank's Balanced Salt Solution (HBSS) with 0.25% (w/v) bovine serum albumin (BSA, fraction V; Sigma). The pancreas was then chopped with sterile scissors into 1 mm fragments and further digested using Type XI collagenase (1 mg/mL; Sigma). The digested tissue was then poured through a 500 µm nylon mesh filter and cultured for 7 days in Ham's F10 medium, supplemented with 10 mmol/L D-glucose, 50 µmol/L isobutylmethylxanthine (ICN, Biomedicals, Montreal, QC, Canada), 0.5% BSA, 2 mmol/L L-glutamine, 3 mmol/L $CaCl_2$, 10 mmol/L nicotinamide (BDH Biochemical, Poole, England), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C, 5% CO_2, and 95% air. The media was changed on days 1, 3, and 5, post-isolation. On day 7, the

islets were collected and aliquots were counted in islet equivalents (IEQ), with 150 µm representing the standard for 1 IEQ[9].

2.2.3 Isolation of Neonatal Porcine SC

Two to three day old male neonatal pigs were anesthetized with isoflurane, and the testicles were removed by dissection and placed in HBSS supplemented with 0.25% (w/v) BSA. The connective tissue lining the testes was removed and the testes were chopped into 1 mm fragments using sterile scissors. These fragments were then subject to digestion with Type XI collagenase (1mg/mL; Sigma) for 7 minutes at 37°C and washed with HBSS. The tissue was further digested with DNase (10 µg/mL, Roche, Laval, QC, CA) and trypsin (25 mg/mL; Roche) in calcium-free medium supplemented with 1 mmol/L ethylene glycol-bis (2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) and 0.5% BSA for 10 minutes at 37°C. Digested tissue was then poured through a 500 µm nylon mesh filter, and washed with HBSS. The Sertoli cells were then co-cultured with NPI 7 days post-isolation in a 1:1000 NPI to SC ratio in supplemented Ham's F10 medium containing 10 mmol/L D-glucose, 50 µmol/L isobutylmethylxanthine, 0.5% BSA, 2 mmol/L L-glutamine, 3 mmol/L $CaCl_2$, 10 mmol/L nicotinamide,100 U/mL penicillin, 100 µg/mL streptomycin, and 10% neonatal pig serum for two days at 37°C, 5% CO_2, and 95% air.

2.2.4 Transplantation of NPI and SC

Following two days of co-culture, NPI and SC were collected and counted, and aliquots of 2000 IEQ and 2 million SC were transplanted under the left kidney capsule of streptozotocin-induced diabetic C57BL/6 mice[9]. Mice were transplanted only after achieving consecutive blood glucose levels of ≥ 17 mmol/L. The aliquots of 2000 IEQ and 2 million SC were aspirated into polyethylene tubing (PE-50) and pelleted by centrifugation. The tubing was then positioned within the subcapsular space of the left kidney, and the tissue was guided in using a micromanipulator syringe. In order to seal the kidney capsule, the puncture is cauterized.

In order to determine the importance of SC being co-transplanted in direct contact with NPI, a separate transplant site experiment was performed. The only modification in the separate site experiment is that NPI and SC were cultured separately, and B6 mice were transplanted with 2000 IEQ under the left kidney capsule and 2 million SC under the right kidney capsule.

Graft function was defined by a state of normoglycemia, or blood glucose levels of less than 10 mmol/L, while graft rejection was defined as the first of three consecutive blood glucose readings which are greater than 10 mmol/L. Survival nephrectomies were performed to ensure that

long-term normoglycemia (>100 days) was graft-dependant, and was validated by a return to the hyperglycemic state.

2.2.5 Monoclonal Antibody Therapy

Transplanted C57BL/6 mice were randomly chosen to receive either no mAb treatment, or intraperitoneal injections of either anti-LFA-1 mAb (KBA; rat IgG2a; prepared as ascites) at 200µg on days 0, 1, 7, and 14 post-transplantation, or the same regimen of isotype control (rat IgG2a; Bioexpress, West Lebanon, NH, USA).

2.2.6 Isolation of Pig Splenocytes

Neonatal pigs were anesthetized with isoflurane, followed by subsequent laparotomy and exsanguination. The spleen was removed by dissection and placed in a 50 mL conical tube containing HBSS with 0.25% (w/v) BSA. The tissue was then poured into a petri-dish and chopped into small segments using sterile scissors. The smaller segments are then rubbed between the coarse ends of frosted microscope slides to release the splenocytes from the capsule and connective tissue. The splenocytes were transferred from the dish to a 50 mL conical tube and placed on ice for 5 minutes to allow large pieces of tissue to settle to the bottom of the tube. The splenocytes were then carefully collected from the conical tube with a pipette and transferred to a new conical tube where red blood cell lysing buffer was then added to the cells to remove the red blood cell population. The remaining lymphocytes are then spun

twice at 1500 RPM at 4°C and resuspended in saline. The number of lymphocytes was determined by staining cells with the exclusion dye Trypan blue, and live cells were counted using a hemocytometer. The lymphocytes were then frozen at -80°C in 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS)[14].

2.2.7 Immunohistological Analysis of Graft Sections

Graft-bearing kidneys were harvested upon graft rejection or at > 100 days post-transplantation and fixed with 10% buffered formalin solution, then embedded in paraffin. Graft sections (5 µm thick) were stained for the presence of insulin secreting cells, SC, and infiltrating immune cells. For the detection of insulin producing cells, the sections were quenched with 10% hydrogen peroxide in methanol, non-specific binding sites were blocked with 20% normal goat serum (Cedarlane, Burlington, ON, CA), followed by a 30 minute incubation with guinea pig anti-porcine insulin antibody (1:1000 dilution, Dako Laboratories, Mississauga, ON, CA). Sections were then incubated for 30 minutes with biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution, Vector Laboratories, Burlington, CA, USA). Avidin-biotin complex/horseradish peroxidase (ABC/HP, Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochroride (DAB, BioGenex, San Ramon, CA, USA) complex undergo a chemical reaction which produce the brown color seen in the sections. The sections are then counterstained with Harris' hematoxylin and eosin[13,14].

For the detection of SC, graft sections were stained for the presence of Müllerian inhibiting substance (MIS) or vimentin. Microwave antigen retrieval was performed on sections in 0.01 mol/L sodium citrate buffer. Sections were quenched with 10% hydrogen peroxide in methanol, non-specific binding sites were blocked with 20% normal goat serum for vimentin staining (Cedarlane), or 20% normal rabbit serum for MIS staining (Cedarlane), followed by a 30 minute incubation with either mouse anti-vimentin (1:100 dilution, Dako) or goat anti-MIS (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were then incubated for 30 minutes with either biotinylated goat anti-mouse secondary antibody for vimentin staining (1:200 dilution, Cedarlane) or biotinylated horse anti-goat secondary antibody for MIS staining (1:200 dilution, Vector Laboratories). Avidin-biotin complex/horseradish peroxidase (ABC/HP, Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochroride (DAB, BioGenex) complex produced the brown color seen in the sections. The sections are then counterstained with Harris' hematoxylin and eosin.

Subpopulations of immune cells were characterized from sections which were cryopreserved in optimum cutting temperature (OCT) solution and snap frozen at -80°C. These sections were fixed in acetone cooled to -20°C for 4 minutes at 4°C. For sections that were stained with foxp3, permeabilization using 0.1% Triton-X 100 Solution (in PBS) was required for 10 minutes. All sections were then blocked with 2% FBS in PBS for 20

minutes and then incubated with an avidin/biotin blocking kit for 10 minutes each to block endogenous biotin or biotin-binding proteins (Vector Laboratories). The sections were then incubated for 45 minutes with rat anti-mouse CD4 (1:500, BD Pharmingen, Mississauga, ON, CA), rat anti-mouse CD8 (1:200, BD Pharmingen), rat anti-mouse CD11b (1:300, BD Pharmingen), or rat anti-mouse CD19 (1:200, BD Pharmingen) antibody, or for 1 hour with rat anti-mouse/rat foxp3 (1:25, eBioscience, San Diego, CA, USA) antibody. Biotinylated rabbit anti-rat IgG (1:200, Vector Laboratories) secondary antibody was then added to the sections for 30 minutes. Similar to paraffin sections, an avidin-biotin complex/horseradish peroxidase (ABC/HP, Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochroride (DAB, BioGenex) complex produced the brown color seen in the sections. All sections were then counterstained with Harris' hematoxylin[14].

2.2.8 Lymphocyte Characterization by Flow Cytometric Analysis

Spleens from transplanted mice, along with naïve controls, were collected at the end of the study in an effort to determine the phenotype of immune cells. Splenocytes were isolated the same as described above for pig splenocytes, and aliquots of 1 million cells per tube were incubated for 30 minutes at 4°C with flourochrome-conjugated primary antibodies (1:100 dilutions; eBioscience, San Diego, CA) specific for numerous immune cell markers. Analysis took place on BD FACS Calibur and BD

LSR II flow cytometers (BD Biosciences, Mississauga, ON, CA), gating only viable spleen cells14.

2.2.9 Detection of Anti-Porcine IgG Antibodies in Mouse Serum by Flow Cytometric Analysis

Anti-porcine IgG levels were detected by flow cytometric analysis of the serum samples of transplanted mice in order to determine the effect of anti-LFA-1 mAb treatment in mice co-transplanted with NPI and neonatal porcine SC on the humoral immune responses. Sera were isolated from blood collected from transplanted B6 mice by heart puncture. Porcine spleen cells (10^6) were obtained from the same pig islet donors and incubated with diluted serum (1:128) for 1 hour at 37°C, 5% CO_2, and 95% air. After washing with 1x PBS, spleen cells were incubated with goat anti-mouse IgG FITC labeled antibody (1:200, Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1 hour at 4°C. Using the BD FACS Calibur flow cytometer (BD Biosciences), the percentage of gated, viable cells bound to antibody was determined. Sera from naïve non-transplanted B6 mice, pig splenocytes incubated without serum or secondary Ab, along with pig splenocytes incubated with secondary antibody absent of mouse serum, were controls for the experiment[14].

2.2.10 Detection of Mouse Cytokines at the Graft Site by Real Time PCR

Sections of graft were snap frozen in liquid nitrogen, and then stored at -80°C. TaqMan RT-PCR (Applied Biosystems, Carlsbad, CA, USA) was used to estimate mRNA levels of the cytokines TGF-β1, IL-10, IFN-γ, and TNF-α at the graft site. Extraction of RNA took place using a QIAGEN RNeasy Mini Kit (QIAGEN Inc., Toronto, ON, Canada). A NanoDrop was used to examine the quality and quantity of RNA. A High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) was utilized in order to produce cDNA from the total RNA. RT-PCR was then performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). TaqMan Fast Universal PCR Master Mix was used for the RT-PCR reaction (Applied Biosystems), while all probe and primer sets were also obtained from Applied Biosystems: Mm00443258_m1, Tnf FAM, Lot P101206-006 H08, Mm01168134_m1 (TNF-α), Ifng FAM, Lot P101206-006 H07 (IFN-γ), Mm01178820_m1, Tgfb1 FAM, Lot P101206-006 H06 (TGF-β1), and Mm00439614_m1, Il10 FAM, Lot P101206-006 H05 (IL-10). All probes are fluorescently labeled with the FAM reporter dye at the 5' end, while the non-fluorescent quencher at the 3' end is a minor groove binder. A non-fluorescent quencher at the 3' end is utilized to minimize the background signal interference with the fluorescent FAM reporter dye at the 5' end. Eukaryotic 18S rRNA was used as a reference gene. Quantification of cytokines was determined utilizing the

comparative cycle threshold (Ct) method. Samples were normalized to the endogenous control 18S rRNA (ΔCt), then normalized to the naïve non-transplanted C57BL/6 mouse kidney control ($\Delta\Delta$Ct), and input into the formula $2^{-\Delta\Delta Ct}$ to calculate the X-fold changes of amplicon occurring between cycles of control samples and experimental test samples.

2.2.11 Detection of Systemic Mouse Cytokines and Serpina3n in Mouse Serum

Mouse cytokines were detected in the serum utilizing a 9-plex mouse cytokine kit, adhering to instructions of the company (Meso Scale Discovery, Gaithersburg, MD, USA). Diluent 4 was dispensed at 25 µL per well in the 96-well Multi-Spot plate, and incubated for 30 minutes with shaking at room temperature. Calibrators and samples were added to each well in duplicate, and incubated for 2 hours with shaking at room temperature. After washing, 25 µL of detection antibody solution was added to each well, and incubated for 2 hours with shaking at room temperature. Finally, following washing, the plate is analyzed on the Sector Imager. The concentration of serpina3n in mouse serum was detected by measuring specific inhibition of granzyme B (GrB) activity in a colorimetric assay. GrB-mediated degradation of Ac-IEPD-pNA (Kaiya Biomedical, Seattle, WA, USA) was conducted for 1 hour at 37°C, in 5% CO_2, and was inhibited by the presence of purified serpina3n (0.6 to 0.0001 mg/mL), which generated a standard curve of A_{405} in relation to serpina3n concentration. A regression curve was generated from the plot,

and was used to calculate the concentration of serpina3n in the serum dilutions. Serum dilutions were carried out in triplicates, and background absorbance was subtracted from equivalent reactions in the absence of GrB.

2.2.12 Adoptive Transfer Experiments

After maintaining long-term normoglycemia (>100 days), 50 million spleen cells isolated from B6 mice co-transplanted with NPI and SC or from naïve non-transplanted control mice were injected into the peritoneum of B6 *rag-/-* mice co-transplanted with NPl and SC from the same pig donor in order to determine the stability of xenograft protection. The remaining splenocytes were characterized by flow cytometry, while sera were also collected in order to determine whether there was a change in the immune response post-adoptive transfer. Blood glucose levels were monitored bi-weekly for 60 days following the adoptive transfer. At 60 days post-injection, or at the time of graft rejection indicated by a return to hyperglycemia, xenografts were harvested and examined for the presence of markers of NPI and SC, along with the presence of immune cells, as described earlier. Splenocytes were isolated and immune cell phenotype of adoptively transferred cells was determined by flow cytometry. Xenograft tissue was preserved for the detection of cytokines by RT-PCR, while sera were collected for detecting the presence of serpina3n, as well as mouse anti-porcine IgG antibodies in comparison to naïve B6 mice controls.

2.2.13 Transplantation of a 2nd Party NPI Xenograft

A number of B6 mice co-transplanted with NPI and SC that maintained normoglycemia for 250 days post-transplantation received a second NPI xenograft transplanted under the right kidney capsule to determine whether xenograft protection could be transferred to a 2nd party NPI donor. Blood glucose levels were monitored bi-weekly for an additional 100 days, at which time the first xenograft transplanted under the left kidney capsule bearing the NPI and SC is removed by survival nephrectomy. The first xenografts were harvested and examined for the presence of markers of NPI and SC, along with the presence of immune cells, as described earlier. Xenograft tissue was preserved for the detection of cytokines by RT-PCR, while sera were collected for detecting the presence of serpina3n, as well as mouse anti-porcine IgG antibodies. Blood glucose levels were monitored to assess the function of the 2nd party xenograft. At 100 days post-transplantation of the 2nd party xenograft, or at the time of graft rejection, the 2nd party xenograft was harvested, and the experiments above were repeated.

2.2.14 Statistical Analysis

Statistical differences for serpina3n data were analyzed using the Mann-Whitney U Test. Statistical differences for flow cytometry, RT-PCR, and systemic cytokine data were analyzed using a 2-way ANOVA and a Bonferroni test for multiple comparisons as a post-hoc analysis. A p-value

≤ 0.05 was considered a statistically significant outcome. Statistical analysis was performed with Graphpad Prism 5.

2.3 RESULTS

2.3.1 Co-transplantation of NPI with Neonatal Porcine SC Combined with Anti-LFA-1 mAb Treatment Protects NPI Xenografts

As previously shown by Ramji *et al*[17], we were able to demonstrate that NPI co-transplanted with SC and treated with anti-LFA-1 mAb were effective at preventing xenograft rejection, as 20/27 (74%) B6 mice achieved long-term xenograft survival (>100 days) (Table 2.1). Similarly, we showed that co-transplanting SC with NPI in the absence of anti-LFA-1 mAb treatment was ineffective at preventing NPI xenograft rejection, as none of the 15 untreated B6 mice achieved normoglycemia. When B6 mice were transplanted with NPI alone in the absence of anti-LFA-1 mAb treatment, all 4 mice also rejected their xenograft, which was a strong indication that SC are unable to protect the NPI xenograft in immune-competent B6 mice without the short-term administration of anti-LFA-1 mAb. In addition to previous findings, we demonstrated that treatment with rat IgG2a isotype control was also ineffective in protecting the NPI xenograft in B6 mice that were co-transplanted with NPI and SC, as none of the 15 mice achieved normoglycemia. In order to determine that the reversal of hyperglycemia was graft-dependant, islet xenograft-bearing kidneys were removed at > 100 days post-transplantation and blood

glucose levels of the recipient mice were monitored. Removal of the islet xenograft bearing kidneys resulted in a return to hyperglycemia in all animals.

Table 2.1: Graft survival in B6 mice transplanted with NPI or NPI and SC and treated with anti-LFA-1 mAb

Treatment	N	Graft Survival (days)	Number of Recipients that Achieved Normoglycemia
NPI	4	0 (x4)	0
NPI+α-LFA-1 mAb	5	0 (x3), >100 (x2)	2
NPI+SC	15	0 (x15)	0
NPI+SC+Isotype	15	0 (x15)	0
NPI+SC+α-LFA-1 mAb	27	>100 (x20), 0 (x7)	20

Mice were transplanted with NPI or NPI plus SC and received either no treatment, Rat IgG2a isotype control (2A3; 200 µg on days 0, 1, 7, 14, post-transplantation), or anti-LFA-1 mAb (KBA; rat IgG2a; 200 µg on days 0, 1, 7, 14, post-transplantation). Blood glucose levels were monitored bi-weekly for a return to normoglycemia.

Examination of the xenografts from protected B6 mice co-transplanted with NPI and SC and treated with anti-LFA-1 mAb by immunohistochemistry showed an abundance in insulin positive beta cells, while treated mice that never achieved normoglycemia had a limited

amount of these insulin producing cells (Figure 2.1). Xenografts from mice that were not treated with a mAb or were treated with a control isotype Ab displayed an absence of insulin producing beta cells (Figure 2.1).

Mice that were treated with anti-LFA-1 mAb also showed the presence of vimentin and MIS positive cells, in both protected and rejected xenografts, indicating the presence of SC (Figure 2.1). This observation seems to indicate that although the NPI xenograft is being rejected, the SC can escape rejection. Interestingly, unlike normal islet morphology, the insulin producing islet cells are found to be dispersed throughout the graft, mostly settling around the tubular structures formed by SC. Mice that were untreated or treated with a control isotype Ab had a small amount of positive staining for vimentin and MIS, however, these cells were found to be very disorganized, failing to form the tubular structures like the SC in the treated groups.

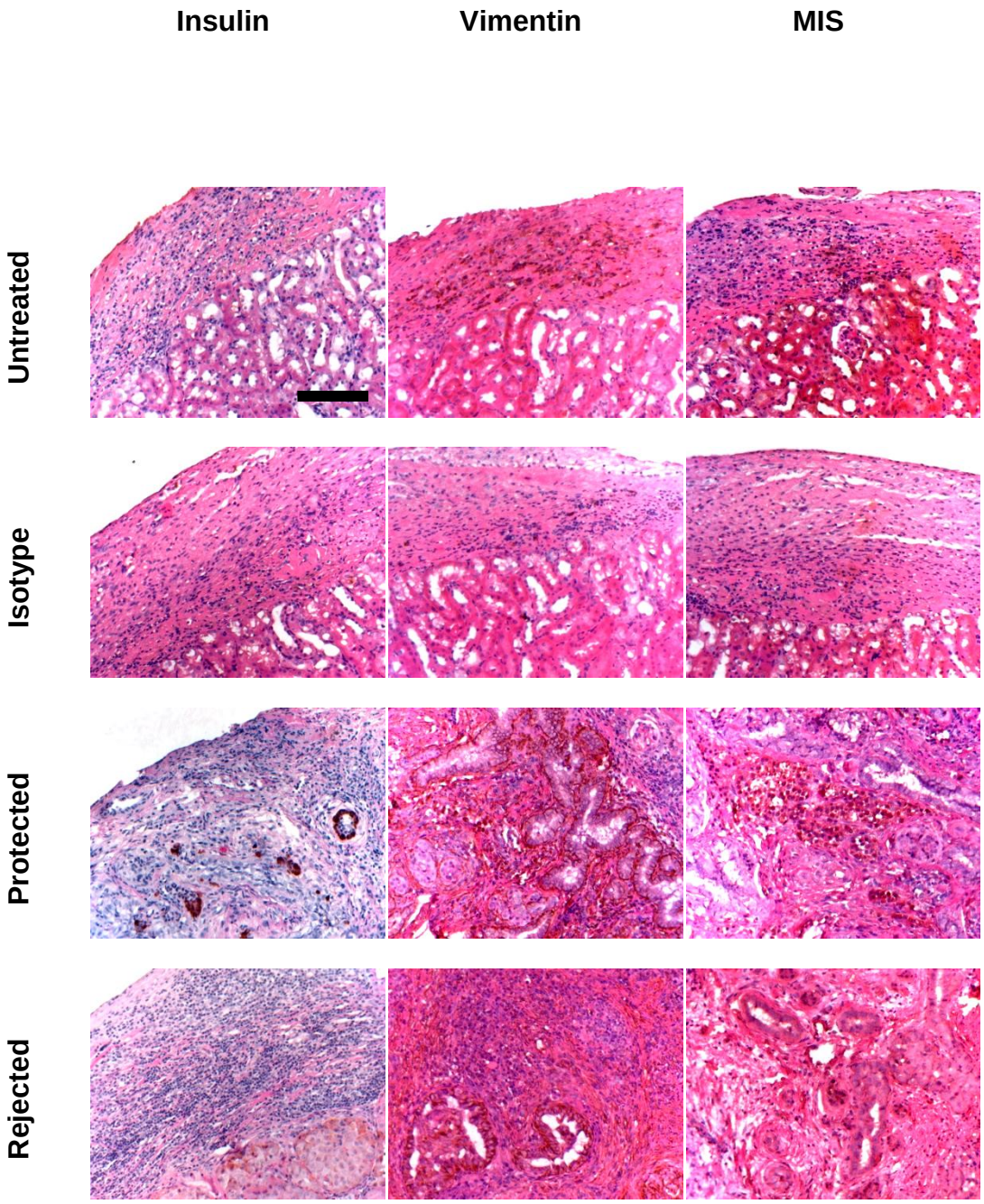


Figure 2.1: Immunohistochemical analysis of xenografts of mice co-transplanted with NPI and SC. Brown staining indicates the presence of insulin, vimentin, or MIS positive cells. Scale bar indicates 400 µm.

2.3.2 Xenografts of Mice Co-transplanted with NPI and SC and Treated with Anti-LFA-1 mAb had Significant $CD4^+$ T cell Infiltrate

Immune cell infiltrates in the grafts were characterized by staining for the presence of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ macrophages (Figure 2.2). $CD4^+$ T cells appear to be the dominant immune cell infiltrate, present in both the protected and rejected xenografts of treated mice. In the rejected grafts, however, it appears that the $CD4^+$ T cells are primarily within the areas where dense immune cell infiltrate is found, while in the protected grafts, the cells are scattered throughout the graft. $CD8^+$ T cells and $CD19^+$ B cells seem to have an increased presence in grafts of rejected mice in areas of dense immune cell infiltrate, while $CD11b^+$ macrophages appear to also play a role in rejection, as they are scattered throughout the rejected xenografts. Interestingly, there appeared to be an increase in the infiltration of $foxp3^+$ T cells in the protected xenografts compared to the rejected, indicating the possibility for T regulatory cells playing a prominent role in the prolonged graft survival of B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment.

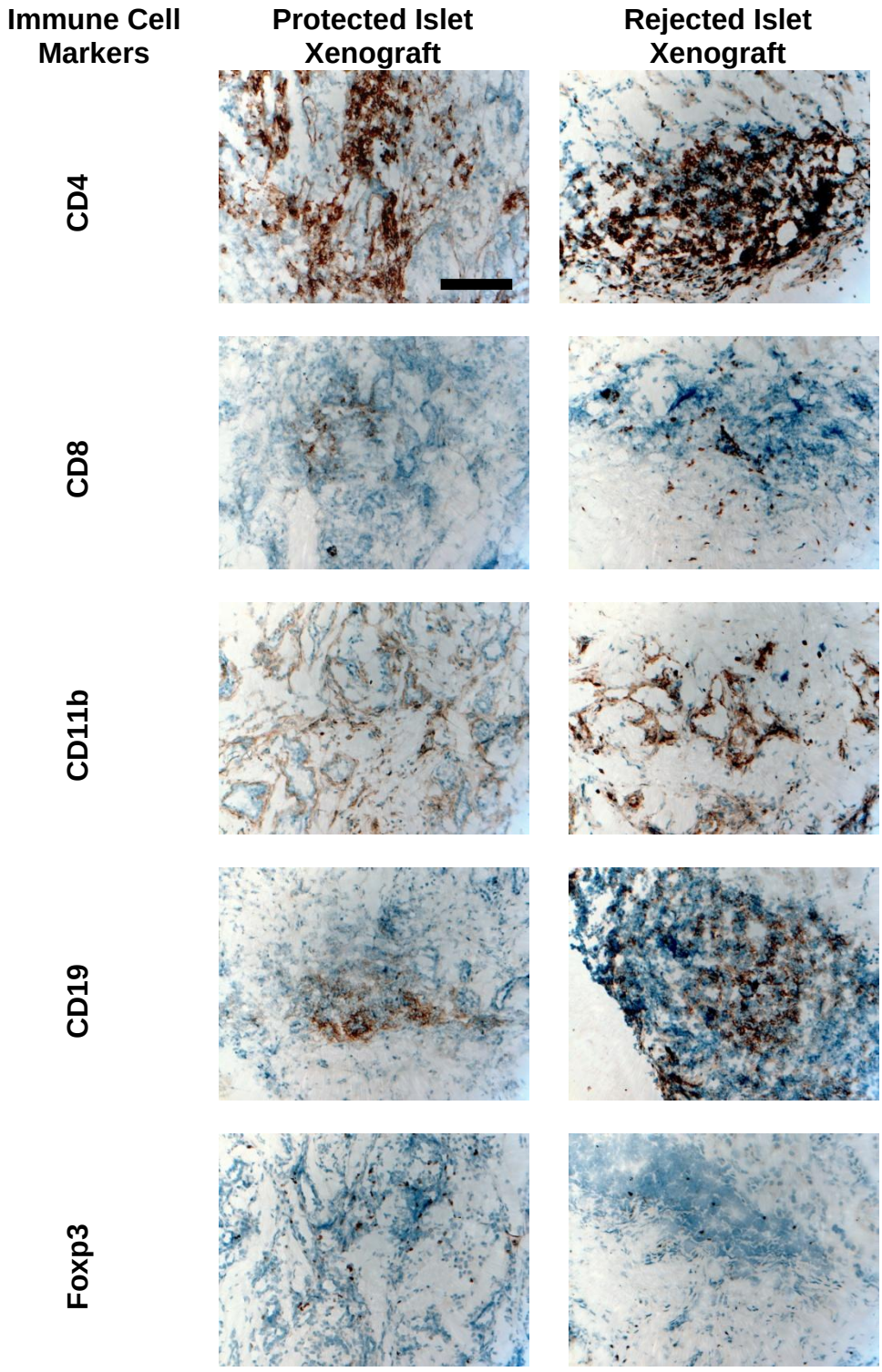


Figure 2.2: Characterization of immune cell infiltrates in the grafts of mice co-transplanted with NPI and SC and treated with anti-LFA-1 mAb. Brown staining indicates the presence of $CD4^+$, $CD8^+$, or $foxp3^+$ T cells, $CD11b^+$ macrophages, and $CD19^+$ B cells. Scale bar indicates 400 µm.

2.3.3 Regulatory T cells are not Solely Responsible for Protection induced by Co-transplantation of NPI and SC Combined with Anti-LFA-1 mAb Treatment

Quantitative RT-PCR assay was performed in order to assess the presence of cytokines at the graft site. Comparable levels of the inflammatory cytokines TNFα and IFNγ were seen in mice with protected and rejected xenografts (Figure 2.3). There was a 2.5 and 3.8 fold increase in protected and rejected grafts respectively, in the anti-inflammatory cytokine TGFβ compared to a naïve kidney control. IL-10 was present at a 451 and 1345 fold increase in protected and rejected grafts respectively, compared to a naïve kidney control. There was a significant increase in the levels of IL-10 present in the grafts of rejected mice as compared to the grafts of protected mice. The immunosuppressive cytokines TGFβ and IL-10 have been shown to play an important role in the function of regulatory T cells, and they have been identified at the graft site in a similar xenograft model[14] where B6 mice were transplanted with NPI alone and treated with anti-LFA-1 mAb and anti-CD154 mAb. Ultimately, regulatory T cells were implicated in preserving long-term graft function in this model[14].

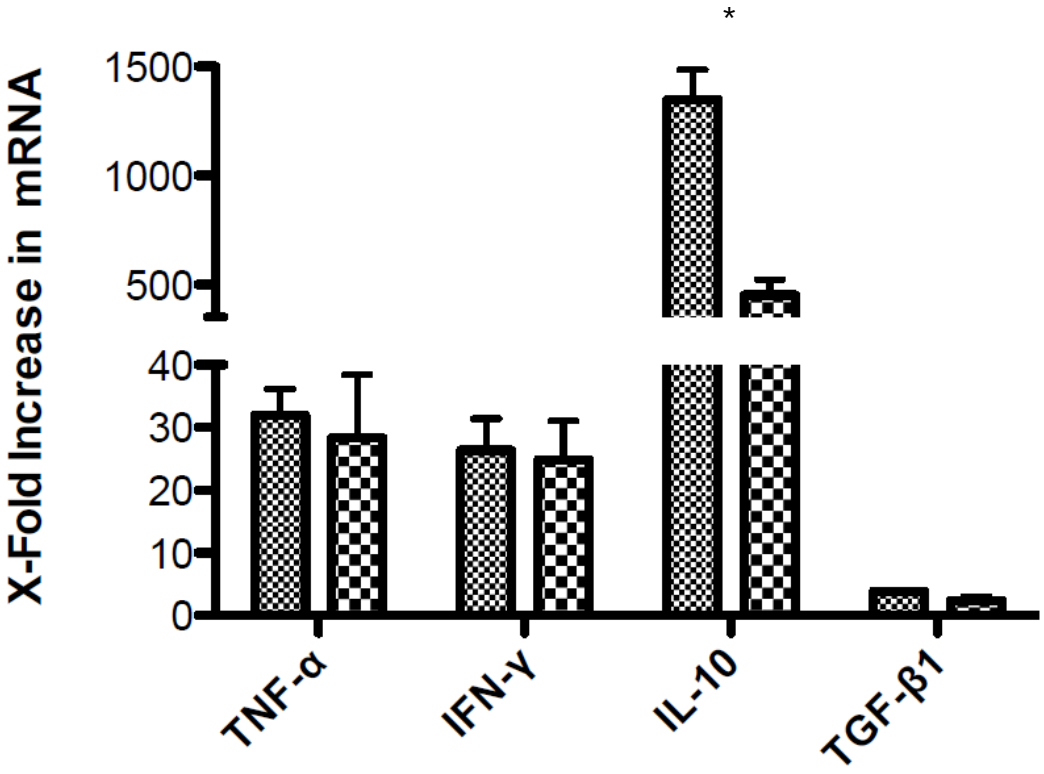


Figure 2.3: Quantitative RT-PCR analysis of cytokines present in the xenograft of B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment. Cytokine levels in the grafts are measured as an X-fold increase compared to a control naïve non-transplanted kidney. The presence of cytokines at the graft site in mice that achieved long-term normoglycemia (Protected:) were compared with the presence of cytokines at the graft site in mice that never achieved normoglycemia (Rejected:).

Flow cytometry was performed on spleen cells of euthanized mice in an effort to examine immune cell phenotype. Identifying changes in the phenotype of different groups of transplanted mice could be important in identifying a mechanism of xenograft protection. There was a significant increase in Foxp3^{+} expressing immune cells in the mice that were co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment regardless of whether the xenograft was protected or rejected compared to that of naïve non-transplanted mice (Table 2.2). It was also noticed that

there was an increase in the percentage of $CD4^+/CD25^+/Foxp3^+$ regulatory T cells in B6 mice with protected xenografts compared to that of B6 mice with rejected xenografts, indicating that these regulatory T cells may play a significant role in graft protection (Table 2.2).

Table 2.2: Phenotype of immune cells isolated from the spleens of naïve non-transplanted B6 mice, and B6 mice that were co-transplanted with NPI and SC and treated with anti-LFA-1 mAb.

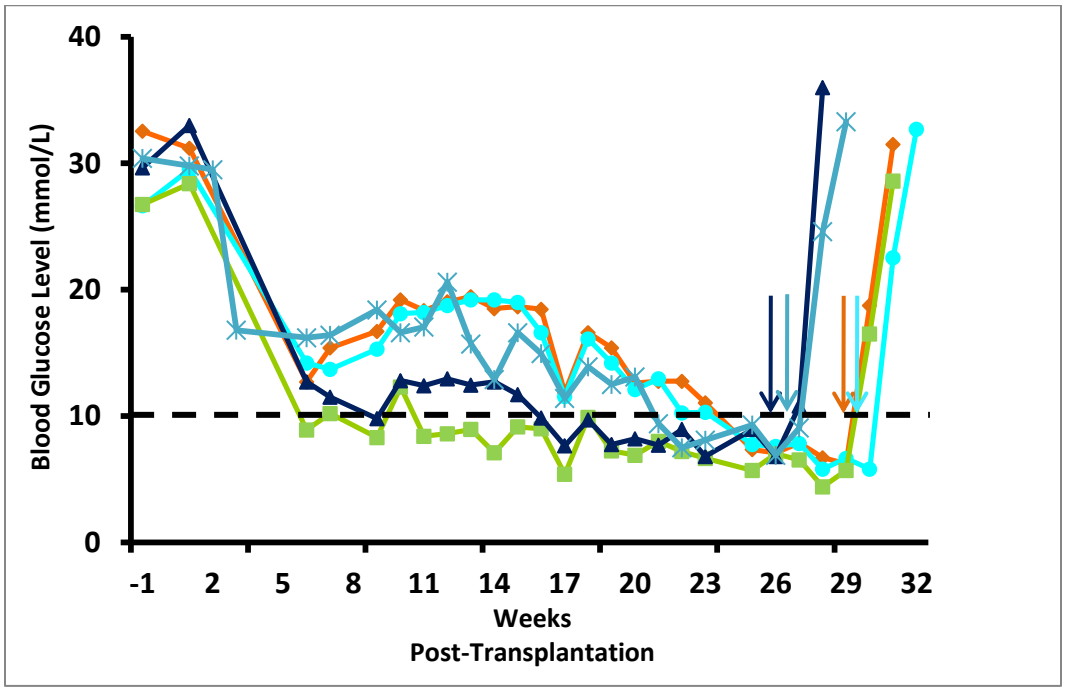
Group	N	Mean Phenotype of Isolated Spleen Cells (%) ± SEM			
		$CD4^+$	$CD25^+$	$Foxp3^+$	$CD4^+/CD25^+/Foxp3^+$
Naive	**4**	**15.98 ± 1.26**	**2.29 ± .012**	**13.25 ± 4.74**	**5.98 ± 0.81**
Protected	**6**	**14.51 ± 0.32**	**2.31 ± 0.17**	**26.27 ± 1.75***	**7.01 ± 0.85**
Rejected	**5**	**13.35 ± 1.65**	**4.42 ± 0.25**	**25.71 ± 5.99***	**3.60 ± 0.49**

Protected mice achieved long-term normoglycemia, while rejected mice never achieved normoglycemia. A more complete table can be found in the Appendix (Table A-1).

In an effort to determine the nature of protection, adoptive transfer experiments were performed on immune deficient B6 *rag-/-* mice which

were co-transplanted with NPI and SC and achieved normoglycemia for >100 days post-transplantation (Figure 2.4). B6 *rag-/-* mice which were reconstituted with spleen cells from naïve non-transplanted B6 mice became diabetic within 12-13 days, with a mean survival time of 12.2 ± 0.1 days post-reconstitution (Figure 2.4A). B6 *rag-/-* mice which were reconstituted with spleen cells isolated from protected B6 mice became diabetic within 13-23 days, with a mean survival time of 18.5 ± 0.6 days post-reconstitution (Figure 2.4B). Immunohistochemical analysis showed that there were no intact islets present in the xenografts of mice whether they were reconstituted with spleen cells from naïve non-transplanted B6 mice or from B6 mice with protected xenografts (Figure 2.5). This was an indication that long-term graft protection was not stable or robust in nature. However, it is apparent that the SC were not rejected, as their tubular structures remain intact in the graft. To confirm, xenografts of B6 *rag-/-* mice that were reconstituted with spleen cells from B6 mice with protected xenografts were stained for the SC markers vimentin and MIS (Figure 2.6). Similar to the immunohistochemical staining demonstrated previously in protected and rejected xenografts (Figure 2.1), a strong vimentin and faint MIS staining was observed.

A)


Blood Glucose Level (mmol/L)
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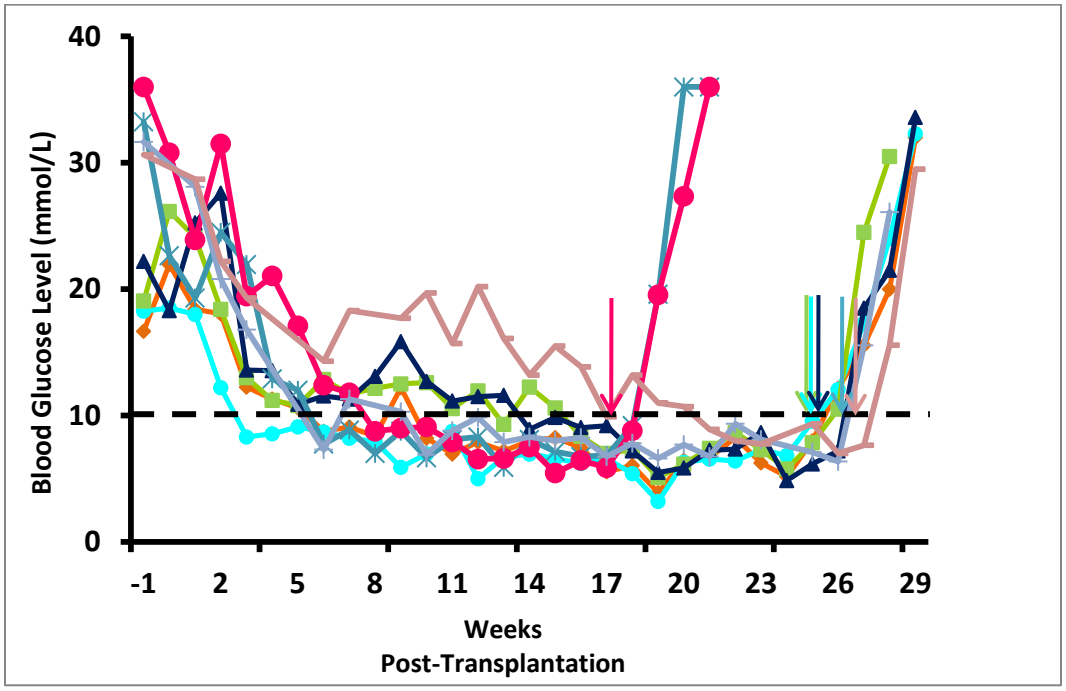
Weeks
Post-Transplantation

B)


Blood Glucose Level (mmol/L)
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Weeks
Post-Transplantation

Figure 2.4: Blood glucose levels of B6 *rag-/-* mice co-transplanted with NPI and SC reconstituted with spleen cells from B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 treatment with protected xenografts. B6 *rag-/-* mice were reconstituted with 50 million spleen cells isolated from either naïve B6 mice (A; n=5) or B6 mice with protected xenografts (B; n=8). Blood glucose levels were monitored bi-weekly for a 60 day follow up period, or until the time of xenograft rejection determined by a return to hyperglycemia.

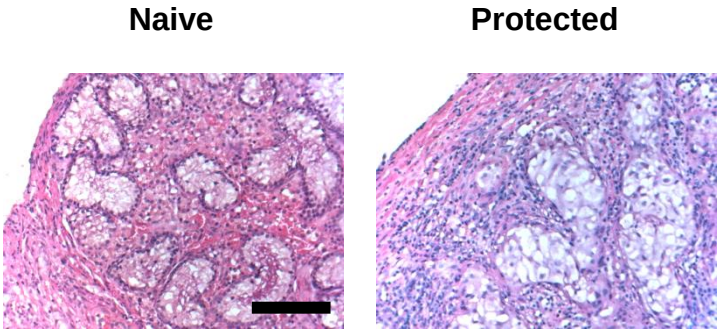

Figure 2.5: Immunohistochemical analysis of xenografts harvested from B6 *rag-/-* mice co-transplanted with NPI and SC and reconstituted with spleen cells from naïve B6 mice or B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment with protected xenografts. Brown staining indicates the presence of insulin positive cells in B6 *rag-/-* mice which were reconstituted with 50 million spleen cells from non-transplanted naïve B6 mice or from treated B6 mice with protected xenografts. Scale bar indicates 400 µm.

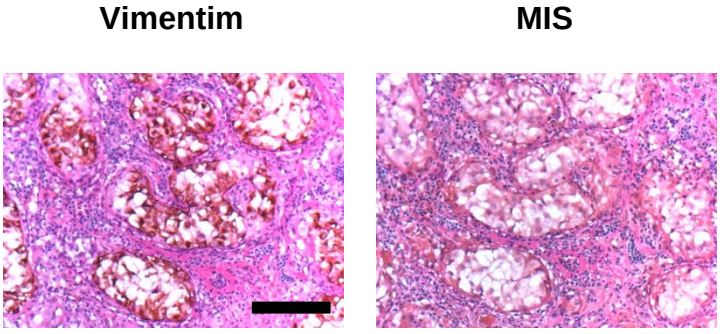

Figure 2.6: Immunohistochemical analysis of xenografts harvested from B6 *rag-/-* mice co-transplanted with NPI and SC and reconstituted with spleen cells from B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment with protected xenografts. Brown staining indicates the presence of vimentin or MIS in B6 *rag-/-* mice which were reconstituted with splenocytes from treated B6 mice with protected xenografts. Scale bar indicates 400 µm.

In an effort to confirm the importance of regulatory T cells in long-term xenograft protection, several mice which achieved long-term normoglycemia were treated with regulatory T cell depleting anti-CD25 mAb. All 8 mice which received injections of anti-CD25 mAb remained normoglycemic for the entire 60 day follow up period (Figure 2.7). This was a strong indication that T regulatory cells are not solely responsible for the long-term xenograft protection seen in B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment.

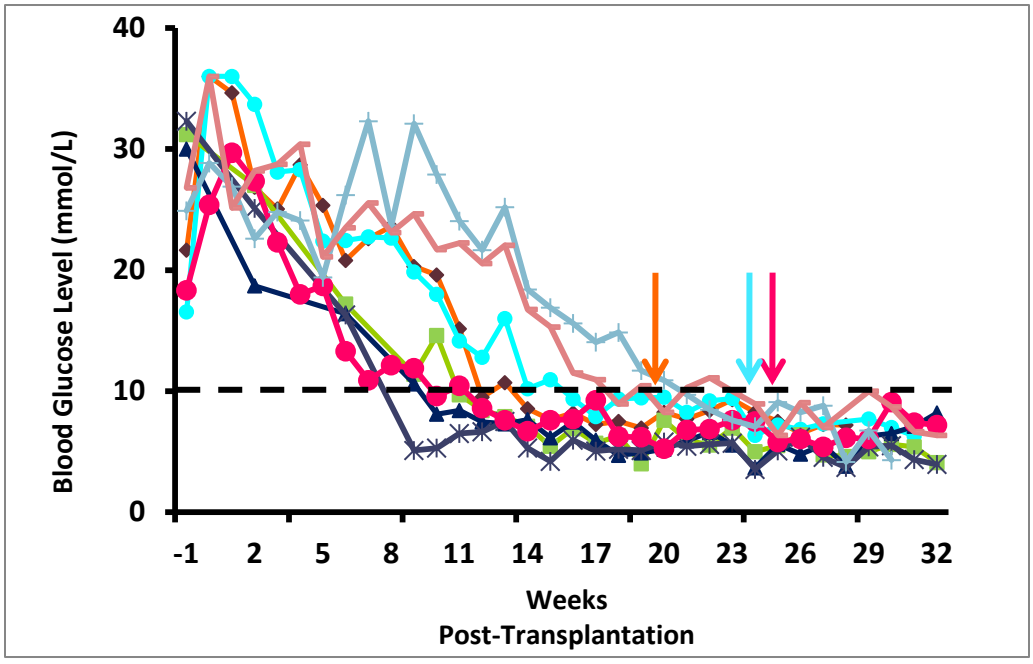

Figure 2.7: Blood glucose levels of protected B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb which were treated with anti-CD25 mAb. The arrows indicate the start of anti-CD25 mAb treatment (orange, blue, and pink arrows represent 1, 2, and 5 mice treated with anti-CD25 mAb, respectively).

2.3.4 Levels of Detected Mouse Anti-Porcine Antibody in the Sera of Mice Co-transplanted with NPI and SC

There appears to be high levels of mouse anti-porcine IgG in protected mice treated with anti-LFA-1 mAb that were normoglycemic prior to euthanization (Table 2.3). In contrast, treated B6 mice that never achieved normoglycemia had low levels of anti-porcine antibodies. One mouse achieved normoglycemia and rejected the xenograft on day 117 post-transplantation. The serum sample harvested from this mouse upon rejection indicated high levels of anti-porcine antibodies, similar to that seen in the protected mice. In mice that received no treatment or rat IgG2a isotype control, there were high levels of antibody production, decreasing in mice euthanized 40 days post-transplantation compared to those euthanized 20 days post-transplantation. Due to the availability of spleen cells required for completion of the experiment, only limited data was collected.

Table 2.3: Percentage of anti-porcine IgG antibodies binding porcine splenocytes in sera of B6 mice co-transplanted with NPI and SC.

Group	Samples			Mean ± SEM
Naïve	1.17	1.48	0.09	0.91 ± 0.42
Untreated (Day 20)	42.34	61.8	50.77	51.64 ± 5.63
Untreated (Day 40)	18.33	18.27	7.72	14.77 ± 3.53
Isotype (Day 20)	61.68	40.88	45.59	49.38 ± 6.30
Isotype (Day 40)	32.81	35.11	7.85	25.26 ± 8.73
Protected (> 100 Days)	92.92	65.07	93.18	83.72 ± 9.33
Rejected (> 100 Days)	9.09	2.99	8.46	6.85 ± 1.94
***Rejected (Day 117)**	91.56			91.56

Mice that received no treatment or isotype control treatment were euthanized on days 20 and 40 post-transplantation. Mice with protected xenografts achieved normoglycemia for >100 days. One mouse that rejected the xenograft achieved normoglycemia and rejected the graft on day 117*, while the remaining mice that rejected xenografts never achieved normoglycemia.

2.3.5 Protection of Xenografts in B6 mice Co-transplanted with NPI and SC and Treated with anti-LFA-1 mAb could not be Extended to 2nd Party NPI xenografts.

A number of protected B6 mice had a 2nd party NPI xenograft transplanted under the right kidney capsule in an attempt to determine whether protection could be extended to an NPI xenograft from a different donor. Three B6 mice that achieved long-term normoglycemia were transplanted with 2000 IEQ under the right kidney capsule. All 3 mice that received a 2nd party NPI xenograft remained normoglycemic for 100 days

post-transplantation of the 2nd NPI xenograft (Figure 2.8). At this time a survival nephrectomy was performed, which the left kidney bearing the initial NPI and SC xenograft was removed. It was then observed that all 3 mice reverted to hyperglycemia, indicating that the 2nd party NPI xenograft was rejected (Figure 2.8). This observation seems to show that the long-term protection rendered to NPI and SC xenografts treated with anti-LFA-1 mAb cannot be extended to an NPI xenograft from a different porcine donor. However, while the 2nd party NPI xenograft was rejected, the 1st party NPI and SC xenograft remained protected, as blood glucose levels remained stable following introduction of the 2nd party graft. There is the possibility that direct contact of the SC with the NPI xenograft is important in long-term protection.

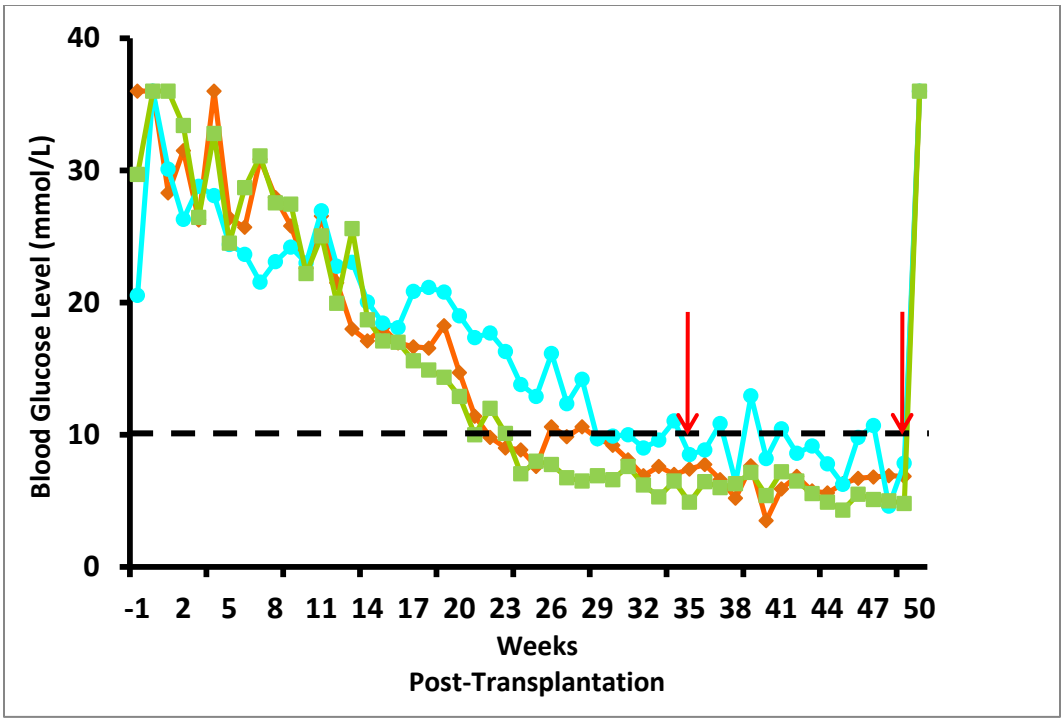

Figure 2.8: Blood glucose levels of B6 mice with protected xenografts transplanted with 2nd party NPI xenografts. The first red arrow represents transplantation of the 2nd party NPI xenograft under the right kidney capsule. The second red arrow represents the nephrectomy of the left kidney bearing the initial NPI and SC xenograft.

2.3.6 Direct Contact of SC with NPI is Necessary to Achieve Long-term Xenograft Protection

A group of B6 mice were transplanted with NPI under the left kidney capsule and SC under the right kidney capsule and treated with anti-LFA-1 mAb in an effort to determine the significance of close contact between the SC and NPI. There were a total of 8 B6 mice that were transplanted at these separate sites, and 4 of these mice achieved normoglycemia (Figure 2.9). There were 2 mice that achieved normoglycemia, but soon after rejected the NPI xenograft. The 2 other mice that achieved

normoglycemia were subject to a survival nephrectomy, at which time they reverted to a diabetic state. Not only does it appear that the efficacy of the treatment is reduced when mice are transplanted at separate sites, but 50% of the mice that achieved normoglycemia experienced only short-term graft protection. This observation seems to indicate that direct contact of SC and NPI through co-transplantation is important in both the efficacy of the treatment as well as the stability of the xenograft protection.

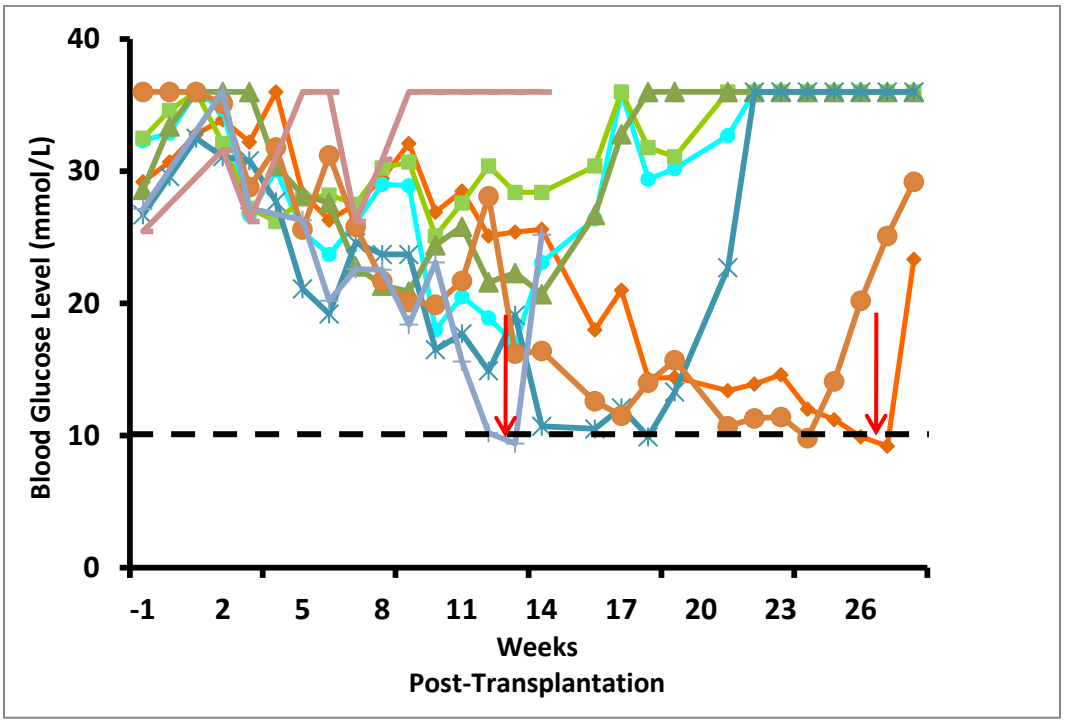

Figure 2.9: Blood glucose levels of B6 mice transplanted with NPI under the left kidney capsule and SC under the right kidney capsule combined with anti-LFA-1 mAb treatment. Red arrows indicate a survival nephrectomy performed on 2 of the 4 mice that achieved normoglycemia.

2.3.7 Cytokines and Secreted Sertoli Cell Products may be Important in Long-term Xenograft Protection

Serum samples of naïve non-transplanted B6 mice and B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment were analyzed for the presence of cytokines and the secreted SC product serpina3n. There was a significant increase in the cytokine IL-12 in B6 mice with protected xenografts compared to that of B6 mice that rejected the xenograft ($p<0.0001$) and naïve non-transplanted B6 mice ($p<0.01$) (Figure 2.10). There were also notable, although insignificant, increases in the cytokines IL-2, IL-4, IL-5, and IL-10 in the mice that had protected grafts compared to the naïve B6 mice. The increases in these cytokines seem to be an indication that helper T cells (T_H) may play an important role in xenograft protection, particularly T_H2 cells. There is also a notable increase in IL-8 in the treated B6 mice whether or not the graft was rejected, compared to naïve B6 mice, which identifies the potential for macrophages to play a role in antigen presentation.

The concentration of serpina3n was also measured in the serum samples obtained from mice using a colorimetric granzyme B specific inhibition assay. The secreted SC product was indentified in the sera of all B6 mice transplanted with NPI and SC at significantly higher levels ($p<0.05$) than that of naïve non-transplanted mice (Figure 2.11). This highlights the possibility of specific SC secreted products playing an important role in the long-term xenograft protection of B6 mice co-

transplanted with NPI and SC and combined with anti-LFA-1 mAb

treatment.

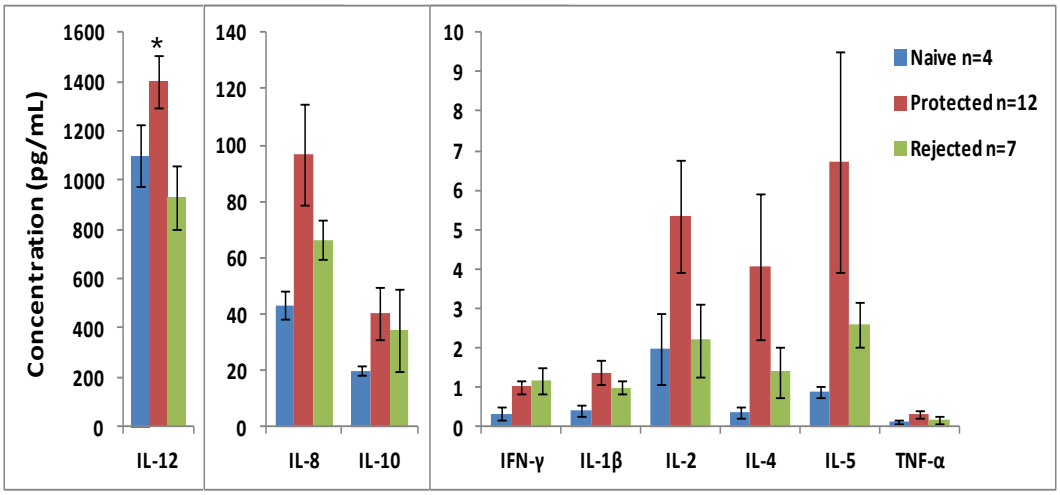

Figure 2.10: Systemic cytokine levels in sera of B6 mice co-transplanted with NPI and SC and treated with anti-LFA-1 mAb compared to naïve non-transplanted mice.

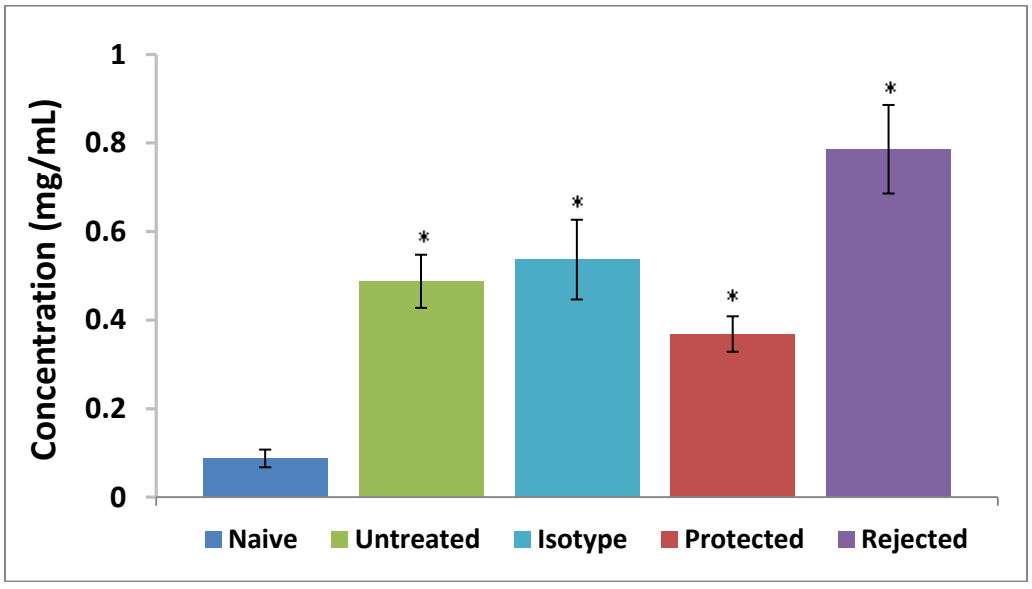

Figure 2.11: Levels of serpina3n in sera of B6 mice co-transplanted with NPI and SC compared to naïve non-transplanted mice. All B6 mice were transplanted with NPI and SC and received no treatment, isotype Ab control, or anti-LFA-1 mAb, with the exception of the naïve non-transplanted control mice.

2.4 DISCUSSION

It has been previously reported that long-term islet xenograft survival can be achieved by co-transplanting NPI and SC into B6 mice combined with anti-LFA-1 mAb treatment[17]. The aim of this study was to confirm these findings and to determine possible mechanisms for the nature of this protection.

Sertoli cells have been shown to be protective to islet grafts in allogeneic[25], xenogeneic (rat and fish to mouse)[26,27], and autoimmune transplant models[28]. However, we showed in this study that co-transplanting NPI and SC without treatment, or with isotype Ab control, is inadequate in preventing NPI xenograft rejection. This was evident as none of the 15 mice that received either no treatment or isotype Ab control treatment achieved normoglycemia, and graft tissue was nonexistent. Other xenograft studies, along with those utilizing NPI and SC[29,30], have also cited the need to incorporate a supplementary approach including induction with anti-lymphocyte serum[26], microencapsulation[27], or the use of a mAb therapy[17].

This study confirmed that SC were able to enhance NPI xenograft survival when co-transplanted in B6 mice combined with anti-LFA-1 mAb treatment as 20 out of 27 (74%) achieved and maintained long-term normoglycemia. This showed marked improvement over the previously

shown success rate of <50% for B6 mice transplanted with NPI alone and treated with anti-LFA-1 mAb[16].

Examination of the xenografts by immunohistochemistry displayed the presence of insulin producing cells along with SC in protected mice. While there was an absence of insulin positive cells in rejected mice, we were able to still identify SC in their trademark tubular configuration[31]. Although a mechanism is undefined, it is possible that secreted immunoprotective SC products such as TGFβ, FasL, clusterin, and serpina3n, in combination with anti-LFA-1 mAb is sufficient to prevent rejection of the SC. We also noticed that islets appeared to scatter along these SC arrangements. There is the possibility that these SC configurations could be harmful to NPI viability and function, and further studies examining this may lead to a clearer understanding of the nature of long-term graft protection.

When observing populations of immune cell infiltrates in transplanted mice, it was apparent that $CD4^+$ T cells were the most abundant in both protected and rejected islet xenografts. In the protected grafts, the $CD4^+$ T cells were distributed throughout the graft, while in the rejected grafts they were found in areas of dense immune cell infiltrate. There seemed to be little difference in the presence of $CD8^+$ T cells and $CD19^+$ B cells in the grafts, appearing slightly more prominent in rejected grafts in areas of dense immune cell infiltrate. There was, however, a notable increase in the presence of $CD11b^+$ macrophages scattered

throughout the grafts of rejected mice. The differing distribution of $CD4^+$ T cells in protected and rejected grafts indicate they may play a role in both long-term graft protection and rejection, while the increase in $CD11b^+$ macrophages presents the possibility that these cells also play a role in rejection. $Foxp3^+$ cells were also seen in xenografts of treated mice, appearing more abundant in protected grafts compared to rejected grafts, indicating a possible role for T regulatory cells in maintaining long-term NPI xenograft protection.

The immune cell phenotypes of B6 mice co-transplanted with NPI and SC and treated with anti-LFA-1 mAb, were compared to that of naïve non-transplanted mice. We observed a significant increase in $Foxp3^+$ cells in treated B6 mice whether or not the xenograft was rejected, compared to that of naïve mice. While not significant, we also noted an increase in $CD4^+/CD25^+/Foxp3^+$ regulatory T cells in mice with protected xenografts in comparison to mice with rejected xenografts. This was an indication that there may be a significant role for T regulatory cells in long-term islet xenograft protection.

Quantitative RT-PCR analysis of graft sections from treated mice showed high levels of IL-10 at the graft site, including a significant increase of IL-10 in mice that rejected the xenograft, while there were comparable low levels of TNFα, IFNγ, and TGFβ, in treated mice whether or not the xenograft was rejected. This contrasts findings from a similar xenograft study which transplanted NPI alone into B6 mice combined with

anti-LFA-1 mAb and anti-CD154 mAb treatment, as they saw increased levels of IL-10 and TGFβ in the grafts of protected mice where T regulatory cells were deemed to be integral in long-term graft protection[14].

An adoptive transfer experiment was performed to gain an understanding of the robustness of xenograft protection. All 8 B6 *rag-/-* mice that were reconstituted with spleen cells from treated B6 mice with protected grafts ended up rejecting the xenografts. These results displayed that long-term graft protection was unstable and was an indication that this protection was unlikely due to the generation of dominant tolerance. In order to confirm the potential significance of T regulatory cells, 8 B6 mice that achieved long-term normoglycemia were treated with depleting anti-CD25 mAb. All 8 mice remained normoglycemic throughout the 60 day follow up period, which provided reasonable evidence to believe that T regulatory cells were not solely responsible for maintaining long-term graft protection. These results, however, do not indicate the importance of T regulatory cells in the induction of long-term graft protection.

Mouse anti-porcine IgG Ab levels were discovered to be very high in mice with protected xenografts, while levels were low in mice that had never achieved normoglycemia. There was one mouse that achieved long-term normoglycemia and rejected the xenograft on day 117 which had similar levels of anti-porcine IgG as mice with protected xenografts. It appears that these levels of antibody production may be due to the

balance of T helper (T_H) cells. It was observed that levels of IL-4, IL-5, and IL-10 were all increased systemically in mice with protected xenografts. It is known that IL-4 is important in establishing an antibody producing T_H2 phenotype, while all three are important cytokines secreted by T_H2 cells[32,33]. While it appears that anti-LFA-1 mAb is unable to inhibit the humoral immune response, SC seem to be supplying additional protection to the NPI xenograft. Also of interest, once islet rejection has occurred, SC remain undamaged in the presence of low levels of anti-porcine IgG Ab. This seems to indicate that in the absence of intact islets, SC may be able to suppress the humoral immune response.

We also showed that long-term graft protection could not be transferred to a 2nd party NPI xenograft. All 3 mice remained normoglycemic for 100 days post-transplantation of the 2nd party NPI xenograft under the right kidney capsule. Following a nephrectomy of the left kidney bearing the initial NPI and SC xenograft, the 3 mice reverted to hyperglycemia, indicating that the 2nd party NPI xenograft was rejected. As the 1st xenograft bearing NPI and SC remained functional throughout the rejection period of the 2nd party NPI xenograft, it is possible that SC provide their protective effect only in close contact with the islet xenograft.

Our results demonstrated that SC appear to require direct contact with the NPI xenograft in order to achieve and maintain long-term graft protection. Four of the 8 mice transplanted with NPI and SC on separate sites achieved normoglycemia. These results are comparable to results

seen in B6 mice transplanted with NPI alone and treated with anti-LFA-1 mAb (<50%)[16], but was inferior to the 74% of mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment that achieved normoglycemia. It was also notable that 2 of the 4 mice were unable to maintain long-term graft protection, rejecting their NPI xenografts on days 127 and 169 post-transplantation, shortly after achieving normoglycemia. These results appear to indicate that the beneficial effects of SC are best represented when SC are in direct contact with the NPI xenograft.

Analysis of cytokines seemed to indicate an importance of T_H2 cells in long-term graft protection. We saw a significant increase in IL-12 as well as an increase in IL-2 in protected mice compared to naïve mice, two cytokines known to be important in differentiation of naïve T cells into T_H0 cells[34,35]. We also noticed a notable increase in IL-4, IL-5, and IL-10 in mice with protected xenografts. As mentioned previously, IL-4 has been shown to be important in differentiating T_H0 cells towards a T_H2 phenotype, and T_H2 cells are known to secrete IL-4, IL-5, and IL-10[32,33]. We also found an increase in IL-8 in protected and rejected mice compared to naïve non-transplanted mice, which suggests macrophages may be important in antigen presentation, and possibly graft rejection[36]. Our studies also demonstrated that the secreted SC product serpina3n was significantly increased in our groups transplanted with NPI and SC, regardless of treatment. Serpina3n has been previously identified to minimize granzyme B mediated apoptosis by a mechanism of irreversible

binding[24]. It is possible that a shift to a T_H2 cell phenotype combined with secreted Sertoli cell products are playing a role in long-term graft protection.

Our studies have demonstrated that the co-transplantation of NPI and SC with combined anti-LFA-1 mAb treatment is an effective therapy for inducing long-term NPI xenograft protection. While T regulatory cells may play a role in protection, possibly by secretion of IL-4 leading to a shift from a T_H1 to a T_H2 cell phenotype, our data supports the idea that T regulatory cells are not solely responsible for maintaining long-term graft protection, while products secreted by SC may share this role.

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CHAPTER 3

GENERAL DISCUSSION AND CONCLUSIONS

3.1 GENERAL DISCUSSION

Insulin was discovered in 1921 and had a major impact in regards to treating individuals suffering from type 1 diabetes[1]. Type 1 diabetes is a chronic disease which is characterized by the inability of β cells in the pancreas to produce insulin[2,3]. In order to control blood glucose levels, individuals are forced with continuously monitoring blood glucose levels, and are subject to regular exogenous insulin injections. Failure to do so can result in serious secondary complications including nephropathy, neuropathy, and hypoglycemic episodes[4,5]. While intensive insulin therapy can provide reasonable control of blood glucose levels, individuals undergoing this mode of treatment have an increased likelihood of experiencing hypoglycemic episodes which can lead to a coma, seizure, or death[5]. However, intensive insulin therapy is a suspect form of treatment for patients diagnosed with brittle type 1 diabetes due to the labile nature of blood glucose levels in these individuals[4,5]. It is of the essence to find a more physiological approach for treating patients with type 1 diabetes in an effort to enhance the quality of life of these individuals.

Numerous potential strategies exist for achieving a more physiological control of blood glucose levels. However, due to success of the Edmonton Protocol, islet transplantation has materialized as a realistic treatment option for individuals with type 1 diabetes. In 2000, it was found that 7/7 patients who received islet allografts achieved and maintained normoglycemia for at least one year post-transplantation[6]. Importantly, control of blood glucose levels showed marked improvement, while a reduction was seen in the occurrence of hypoglycemic episodes[6]. While islet transplantation has shown promise in this study, barriers to its widespread application exist. The shortage of cadaveric pancreas donors and the need for continuous use of immunosuppressive drugs currently limits the eligible patients for this procedure to those suffering from brittle type 1 diabetes[6,7]. In an effort to make islet transplantation a viable option for more patients, it is necessary to identify an alternative source of donor islets, along with a safe and effective anti-rejection strategy.

A possible solution to overcoming the shortage of donor islets includes the utilization of xenogeneic tissue as an alternative source. Porcine tissue represents a promising candidate as a source of islets as pigs breed rapidly, litter sizes are large, gestational time is short, they can be housed in a pathogen-free environment, and the potential exists to generate transgenic pigs that are genetically altered for producing safer tissue for transplantation[7-9]. It is also notable that porcine islets are similar to human islets morphologically and physiologically, while porcine insulin

has successfully been used in regulating blood glucose levels in patients with type 1 diabetes for decades[7]. While both neonatal and adult porcine islets have been successful establishing euglycemia in nonhuman primates[10-12], neonatal islets appear to provide the best overall solution. Neonatal pigs provide cost benefit due to reduced housing times, while NPI are more resistant to hypoxia-induced apoptosis, display growth potential, and appear less immunogenic[13,15-16]. A major limitation when considering xenogeneic tissue as an alternative source of islets is the potent cellular immune response of the host immune system, which is capable of destroying xenograft tissue[17]. As an alternative to harmful immunosuppressive drugs, finding short-term therapies which can induce tolerance to donor tissue with minimal toxicity is imperative to the future clinical application of xenotransplantation.

A potential solution involves the use of biologic agents which target pathways integral in T cell activation and function. The use of anti-LFA-1 mAb is a therapy in particular which has been investigated due to the importance of leukocyte function-associated antigen-1 (LFA-1) in adhesion, activation, and migration of leukocytes[18]. Interaction of LFA-1 with one of its ligands, inter-cellular adhesion molecule-1 (ICAM-1), has been shown to provide a co-stimulatory signal to T cells, optimize contact between T cells and the APC, along with lowering antigen requirements necessary for T cell activation[18-21]. Blockade of this interaction has the potential to promote tolerance induction. While anti-LFA-1 mAb was

shown to be highly efficacious in preventing concordant rat to mouse islet xenograft rejection, it was found to be only moderately effective in preventing discordant pig to mouse islet xenograft rejection[23]. In our study, we looked at augmenting this mAb therapy by co-transplanting NPI and SC in an effort to enhance the potential of promoting long-term xenograft survival.

Sertoli cells have exhibited an ability to prevent rejection of transplanted graft tissue both inside and outside the testicular environment, attributed to the secretion of numerous immunoprotective molecules. Prolonged graft survival has been observed in both allograft and autoimmune models with co-transplanted islets and SC[30,31]. However, in order to prevent graft rejection in a xenograft model, SC seem to require additional inductive immunosuppression[32,33].

Previous findings have shown that short-term administrations of anti-LFA-1 mAb combined with co-transplantation of NPI and SC may have the potential to induce long-term NPI xenograft protection in a B6 mouse model[34]. It therefore became our directive to determine potential mechanisms responsible for long-term NPI xenograft protection. Anti-LFA-1 mAb has been shown to interfere with T cell activation and the potential to inhibit humoral responses[18,22,35], while SC are known to secrete numerous immunosuppressive products such as TGFβ and serpina3n[29,36]. Moreover, anti-LFA-1 mAb, in combination with other mAbs, has demonstrated the ability to facilitate long-term protection of NPI

xenografts by way of T regulatory cells[35]. There appears to be an important role for TGFβ in the development of foxp3$^+$ T regulatory cells, thus the possibility for induction of T regulatory cell mediated tolerance towards NPI and SC xenografts seems plausible in our combination therapy.

We have confirmed in our study that the combination of SC and anti-LFA-1 mAb treatment is efficacious in inducing and maintaining long-term normoglycemia as 20/27 (74%) mice achieved prolonged NPI xenograft survival of >100 days. In contrast, none of the untreated (0/15) or isotype Ab control (0/15) mice co-transplanted with NPI and SC achieved long-term normoglycemia, while only 2/5 (40%) mice transplanted with NPI alone and treated with anti-LFA-1 mAb achieved normoglycemia. Histological analysis of xenografts showed the presence of insulin, vimentin, and MIS positive cells in mice with protected grafts indicating the presence of β cells and SC at the studies endpoint. Conversely, mice with rejected xenografts displayed an absence of insulin producing β cells, yet showed the presence of vimentin and MIS demonstrating the presence of SC at the studies endpoint. Further histological analysis of immune cell infiltrates indicated that CD4$^+$ T cells were most abundant in the xenografts of treated mice, and were evident regardless of whether rejection of the graft occurred. There was a decreased presence of CD8$^+$ T cells, CD19$^+$ B cells, and CD11b$^+$ macrophages in the xenograft, while there appeared to be slight increases

in these immune cells present in rejected xenografts. Interestingly, the apparent increase in foxp3$^+$ cells in protected grafts compared to rejected grafts potentially identifies a role for T regulatory cells in maintaining long-term NPI xenograft rejection.

Flow cytometry of spleen cells further supported a potential role for T regulatory cells in graft protection, as there was not only a significant increase in foxp3+ cells in treated mice compared to naïve controls, but also a notable increase in CD4$^+$/CD25$^+$/Foxp3$^+$ T regulatory cells in mice with protected mice compared to mice with rejected grafts. While this increase in T regulatory cells in mice with protected xenografts was not significant, the previously mentioned findings provided rationale to further investigate the importance of T regulatory cells in long-term NPI xenograft protection.

Examination of quantitative RT-PCR assays demonstrated only low levels of increased TGFβ in treated mice compared to naïve controls, while there was a significant increase in the levels of IL-10 in mice with rejected xenografts compared to mice with protected xenografts. Previous studies have shown IL-10-producing CD4$^+$ T cells to exert antitumor effects[14]. Interestingly, these CD4$^+$ T cells did not appear to share characteristics of typical T_H2 cells due to the absence of IL-4 detected in tumor-infiltrating lymphocytes, and instead appeared to share the properties of T_R1 cells, which generally reduce rather than promote inflammatory responses[14]. Due to the potential of IL-10 to have

proinflammatory effects in some autoimmune diseases raises question as to whether IL-10-producing CD4+ T cells may play an active role in NPI xenograft rejection. Thus, exogenous IL-4 injections at the time of transplantation in an effort to promote a T_H2 cell phenotype may improve the efficacy of our therapy.

Adoptive transfer experiments indicated the lack of a stable robust graft protection as all of the B6 *rag-/-* mice reconstituted with spleen cells from treated mice with protected xenografts were unable to maintain long-term graft protection. Furthermore, all of the treated mice with protected xenografts that underwent depletion of T regulatory cells by administration of anti-CD25 mAb maintained normoglycemia. A decrease in anti-porcine antibodies was detected in these mice compared to other mice with protected xenografts that were not treated with anti-CD25 mAb, indicating a potential role for T regulatory cells in modulating the antibody response in recipient mice. Together these results indicate that T regulatory cells are not solely responsible for the long-term graft protection seen in B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment. It is possible, however, that T regulatory cells in combination with transient anti-LFA-1 mAb treatment are critical to induction of NPI xenograft protection, and depletion of these cells near the time of transplantation may be helpful in determining their importance.

Mouse anti-porcine IgG Ab levels were discovered to be very high in mice with protected xenografts, while levels were low in mice that had

never achieved normoglycemia. There was one mouse that achieved long-term normoglycemia and rejected the xenograft on day 117 which had similar levels of anti-porcine IgG as mice with protected xenografts. It appears that these levels of antibody production may be due to the balance of T helper (T_H) cells. Previous studies have implicated anti-LFA-1 mAb in playing a role in promoting a T_H2 cell phenotype[41-43], while it was observed that levels of IL-4, IL-5, and IL-10 were all increased in mice with protected xenografts. It is known that IL-4 is important in establishing an antibody producing T_H2 phenotype, while all three are important cytokines secreted by T_H2 cells[32,33]. Future studies utilizing our therapy in IL-$4^{-/-}$, IL-$5^{-/-}$, and IL-$10^{-/-}$ mice could be beneficial in identifying the importance of these individual cytokines in long-term NPI xenograft protection. Also of interest, once islet rejection has occurred in mice treated with anti-LFA-1 mAb, SC remain undamaged in the presence of low levels of anti-porcine IgG Ab at > 100 days post-transplantation. In mice that were untreated or treated with isotype Ab control it was demonstrated that antibody levels were also quite high at 20 days post-transplantation, while the levels decreased at 40 days post-transplantation. Histological analysis of these grafts revealed an absence of intact islets and SC. This indicated that while anti-LFA-1 mAb appears unable to inhibit the humoral immune response, it is necessary to prevent initial rejection of SC. As there appears to be intact SC in mice that had protected and rejected the xenografts, and levels of anti-porcine antibody varied greatly between

these two groups of mice, it appears that the increased humoral activity is not due to the presence of SC. Future studies will need to examine whether the increased humoral activity in mice with protected xenografts is a sign of ongoing islet rejection. Although previous results indicate that the humoral response is not a major concern in NPI xenograft rejection[7], transplanting mice with SC alone combined with anti-LFA-1 mAb treatment could provide further insight as to whether SC have the potential to suppress the humoral immune response in the absence of intact NPI.

Introduction of a 2nd party NPI xenograft under the opposite kidney capsule of treated B6 mice that achieved long-term normoglycemia was insufficient at maintaining normoglycemia upon removal of the initial NPI and SC xenograft. These results indicated that graft protection induced in B6 mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment could not be transferred to a 2nd party NPI xenograft. This is a significant finding, as multiple transplants are often required in a clinical setting. It appears that an additional mAb and SC regimen would be required in order to prevent rejection of the 2nd party NPI xenograft, potentially limiting the clinical applicability of this therapy.

When NPI and SC were transplanted separately under opposite kidney capsules, the beneficial effects of SC appear to be lost, as efficacy of the treatment was reduced to levels seen in B6 mice transplanted with NPI alone combined with anti-LFA-1 mAb. Four of the 8 mice transplanted with NPI and SC on separate sites achieved normoglycemia.

These results are comparable to results seen in B6 mice transplanted with NPI alone and treated with anti-LFA-1 mAb (<50%)[16], but was inferior to the 74% of mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment that achieved normoglycemia. These results appear to indicate that the beneficial effects of SC are best represented when SC are in direct contact with the NPI xenograft.

Levels of serpina3n were also found to be significantly increased in mice transplanted with NPI and SC compared to naïve controls. However, there was no significant difference in serpina3n levels whether or not the mice rejected the xenografts. While it remains unclear what impact serpina3n has in graft protection, further examination of secreted SC products may lead to a clearer understanding of the mechanism of long-term NPI xenograft protection. While $CD4^+$ T cells have been shown to express granzyme B and perforin in a CMV infection model[44], this has yet to be examined in our xenograft model. It is possible that the immune system identifies the NPI xenograft as an infection, thus, future studies aimed at exploring this potential in $CD4^+$ T cells may be beneficial in understanding the protective role of SC in our therapy.

In light of our findings, a number of important questions still exist, including: i) further elucidating the mechanisms of long-term graft protection, ii) examining the mechanisms of graft rejection, including a possible role for SC and iii) evaluating the efficacy and safety of this

treatment in more clinically applicable models, including autoimmune and large animal transplant models.

While a number of possible mechanisms were identified in our study, it remains important to assess the nature of graft protection. Even though T regulatory cells were found to be not solely responsible for maintaining long-term xenograft protection in our model, further examination of the role that T regulatory cells may play in induction of graft protection could be valuable. Depletion of T regulatory cells by administration of anti-CD25 mAb closer to the time of transplantation may provide further evidence on the importance of T regulatory cells in induction of NPI xenograft protection. Evaluation of these studies may provide further insight as to why some xenografts are rejected and unable to achieve long-term graft protection. Our data further suggests that T_H2 cells may play a significant role in maintaining long-term NPI xenograft protection, and manipulating the balance of these T_H cells may help develop the mechanism of graft protection. Depletion of IL-4 with a neutralizing Ab in mice with protected xenografts may provide evidence as to the importance of this T_H2 cell phenotype in maintaining long-term xenograft protection. Additionally, the secreted SC product serpina3n was present the serum of mice transplanted with NPI and SC. While there was no significant increase in serpina3n in mice with protected xenografts compared to mice with rejected xenografts, it may be valuable to attempt to neutralize serpina3n both at the time of transplantation as well as once

the mice have achieved stable long-term normoglycemia in an attempt to determine the importance of this secreted SC product in NPI xenograft protection. Further investigation into the presence of other secreted SC products such as FasL and clusterin has the potential to aid in development of the mechanism of graft protection. Thus, further elucidation of the mechanisms of graft protection is important as it may allow us to improve the overall efficacy of this treatment.

While SC appear to protect themselves, the possibility exists that SC may also be destructive to the health of the islets. We found that some immune deficient B6 *rag-/-* mice had difficulty achieving normoglycemia, while B6 *rag-/-* mice transplanted with the islets from the same donor did not. This may be due to the SC competing for space and nutrients with the NPI, or possibly SC secretions of FasL leading to destruction of Fas-expressing NPI[31,37]. Previous studies have identified that the ratio of islets and SC transplanted are important in a syngeneic autoimmune model and a rat allograft model[30,31]. Supplementary studies examining the potentially harmful effects of SC, and determining whether a more optimal ratio of NPI and SC exists in a xenograft model could be essential in enhancing the efficacy of this treatment.

Finally, it is necessary to evaluate the efficacy and safety of these treatments in a more clinically relevant transplant model. While anti-LFA-1 mAb was used commonly in humans to treat psoriasis[38], some concern has arisen due to the development of the rare disease progressive

multifocal leukoencephalopathy (PML) associated with long-term use of the drug[38-40]. In the event that the use of anti-LFA-1 mAb becomes no longer realistic, finding antibodies that target the same interaction without the harmful side effects will be necessary for clinical application in islet transplantation. Furthermore, transplantation of SC into male and female patients has the potential to disrupt the hormonal balance in the human body through the manipulation of testosterone and follicular stimulating hormone. Thus, detailed studies should be conducted to determine potential unwanted side effects of transplanting SC in small and large animal models in order to develop a better understanding of the clinical applicability of this treatment. Evaluating the potential efficacy of this treatment in a more relevant model to type 1 diabetes in humans, such as an autoimmune diabetic mouse model, would also be beneficial.

Overall, we have demonstrated in our study that short-term administration of anti-LFA-1 mAb is effective at prolonging NPI xenograft survival when co-transplanted with SC. We have determined that T regulatory cells are not solely responsible for maintaining long-term NPI xenograft protection, and that this protection is not very stable and/or robust. We also demonstrated the potential for T_H2 cells and secreted SC products to contribute to maintaining long-term xenograft protection. Therefore, further studies must be performed to investigate the proposed potential mechanisms, to explore the optimal ratio of NPI and SC, and to

further determine the safety and efficacy of the treatment, especially in additional animal models.

3.2 Conclusion

The potential for islet transplantation to restore precise physiological control of blood glucose levels makes it one of the most promising strategies for treating patients with type 1 diabetes. The ability to eliminate the need for daily exogenous insulin injections and continuous monitoring of blood glucose levels, while preventing the occurrence of harmful secondary complication provides motivation to investigate improvements to this therapy to permit widespread application.

While the use of xenogeneic NPI has become an attractive alternative source for overcoming the shortage of donor islet tissue for transplantation, clinical use requires further studies to evaluate the potential of developing safe and effective strategies to prevent xenograft rejection.

In our study we demonstrated that combining the short-term administration of anti-LFA-1 mAb with the co-transplantation of NPI and SC can be effective at preventing NPI xenograft rejection. We determined that long-term xenograft protection is not solely due to T regulatory cells, and that T_H2 cells and/or secreted SC products may play a contributing role. Future studies will be important in further investigating the

mechanism of induction and maintenance of long-term NPI xenograft protection, and may lead to providing insight on ways to improve not only this therapy, but possibly other therapies utilizing NPI as an alternative source of donor tissue. We hope that these data can provide a foundation for future studies which can contribute to the development of safer anti-rejection therapies for NPI islet xenografts, with the desire to ultimately improve the quality of life of individuals suffering from type 1 diabetes.

3.3 References

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APPENDIX

Table A-1: Phenotype of immune cells in mice co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment compared to naïve non-transplanted controls.

Immune Cell Phenotype	*Mean + SEM Percentage of Immune Cells*				
	Naive	**Untreated**	**Isotype**	**Protected**	**Rejected**
	N = 4	**N = 5**	**N = 5**	**N = 6**	**N=5**
CD4	15.98 ± 1.26	14.64 ± 1.85	18.20 ± 2.55	14.51 ± 0.32	13.35 ± 1.65
CD8	12.09 ± 0.76	11.61 ± 0.53	13.20 ± 0.72	9.27 ± 0.64	13.67 ± 1.33
CD19	61.78 ± 2.98	62.20 ± 3.26	58.95 ± 3.29	59.31 ± 2.48	57.05 ± 4.25
CD25	2.29 ± 0.12	3.36 ± 0.33	3.10 ± 0.13	2.31 ± 0.17	4.42 ± 0.25
CD44	96.88 ± 0.08	96.41 ± 0.06	95.17 ± 0.47	97.41 ± 0.26	95.68 ± 0.69
CD62L	75.87 ± 1.67	56.38 ± 5.77	45.60 ± 5.54	67.14 ± 1.65	51.84 ± 4.06
CD69	13.00 ± 1.54	30.86 ± 1.78	28.28 ± 2.16	4.40 ± 0.39	38.95 ± 6.77
PD1	22.53 ± 8.29	24.65 ± 7.02	24.04 ± 9.6	12.72 ± 2.18	62.36 ± 3.18
CTLA4	3.56 ± 1.00	6.36 ± 2.01	6.42 ± 2.47	2.17 ± 0.50	3.42 ± 1.17
BTLA	63.75 ± 1.73	70.26 ± 2.6	64.53 ± 3.32	68.43 ± 1.59	68.21 ± 2.45
FoxP3	13.25 ± 4.74	6.27 ± 1.16	5.96 ± 1.23	26.27 ± 1.75	25.71 ± 5.99
GITR	34.63 ± 2.20	27.10 ± 1.62	31.38 ± 2.12	28.76 ± 1.88	34.83 ± 2.61
CD4/CD25	1.77 ± 0.10	2.35 ± 0.23	2.17 ± 0.14	1.67 ± 0.17	2.21 ± 0.27
CD4/CD44	17.43 ± 1.53	16.29 ± 2.97	18.01 ± 2.09	15.14 ± 0.70	12.98 ± 1.38
CD4/CD62L	13.08 ± 1.40	10.42 ± 1.07	12.06 ± 1.46	9.44 ± 0.69	6.51 ± 0.52
CD4/CD69	3.58 ± 0.22	6.97 ± 1.38	8.14 ± 1.76	1.92 ± 0.28	6.66 ± 1.39
CD4/PD1	4.75 ± 0.39	3.03 ± 0.37	2.97 ± 0.65	5.15 ± 0.26	7.04 ± 0.93
CD4/CTLA4	1.82 ± 0.18	0.99 ± 0.24	1.14 ± 0.40	1.22 ± 0.13	1.05 ± 0.24
CD4/BTLA	1.32 ± 0.18	0.98 ± 0.16	0.95 ± 0.24	1.55 ± 0.44	1.65 ± 0.25
CD4/FoxP3	4.68 ± 0.25	1.57 ± 0.37	1.70 ± 0.48	6.16 ± 0.27	4.08 ± 0.96
CD4/GITR	14.77 ± 1.34	12.92 ± 1.49	15.26 ± 1.79	12.45 ± 0.52	13.19 ± 1.28
CD8/CD25	0.16 ± 0.03	0.15 ± 0.02	0.17 ± 0.05	0.21 ± 0.07	0.19 ± 0.06
CD8/GITR	10.00 ± 1.29	10.58 ± 0.45	11.90 ± 0.53	7.79 ± 0.70	12.92 ± 1.89
CD25/FoxP3	0.88 ± 0.12	0.61 ± 0.09	0.56 ± 0.08	1.09 ± 0.08	1.26 ± 0.34
CD25/GITR	1.51 ± 0.17	2.33 ± 0.22	2.21 ± 0.14	1.59 ± 0.23	3.42 ± 0.29
CD4/CD44/CD62L	73.41 ± 1.66	52.65 ± 3.08	62.26 ± 2.75	54.40 ± 2.50	65.63 ± 3.63
CD4/CD25/FoxP3	5.98 ± 0.81	1.75 ± 0.34	1.27 ± 0.20	7.01 ± 0.85	3.60 ± 0.49
CD4/CD25/PD1	5.14 ± 1.33	5.56 ± 1.08	4.23 ± 1.01	4.99 ± 0.50	7.79 ± 0.89
CD4/CD25/CTLA4	3.35 ± 1.24	1.93 ± 0.51	2.01 ± 0.72	4.15 ± 1.67	2.18 ± 0.72
CD4/CD25/BTLA	1.34 ± 0.44	0.74 ± 0.26	0.67 ± 0.26	1.33 ± 0.42	0.73 ± 0.20
CD4/CD25/GITR	10.88 ± 1.66	17.63 ± 1.07	14.46 ± 1.12	10.46 ± 0.57	15.64 ± 0.76

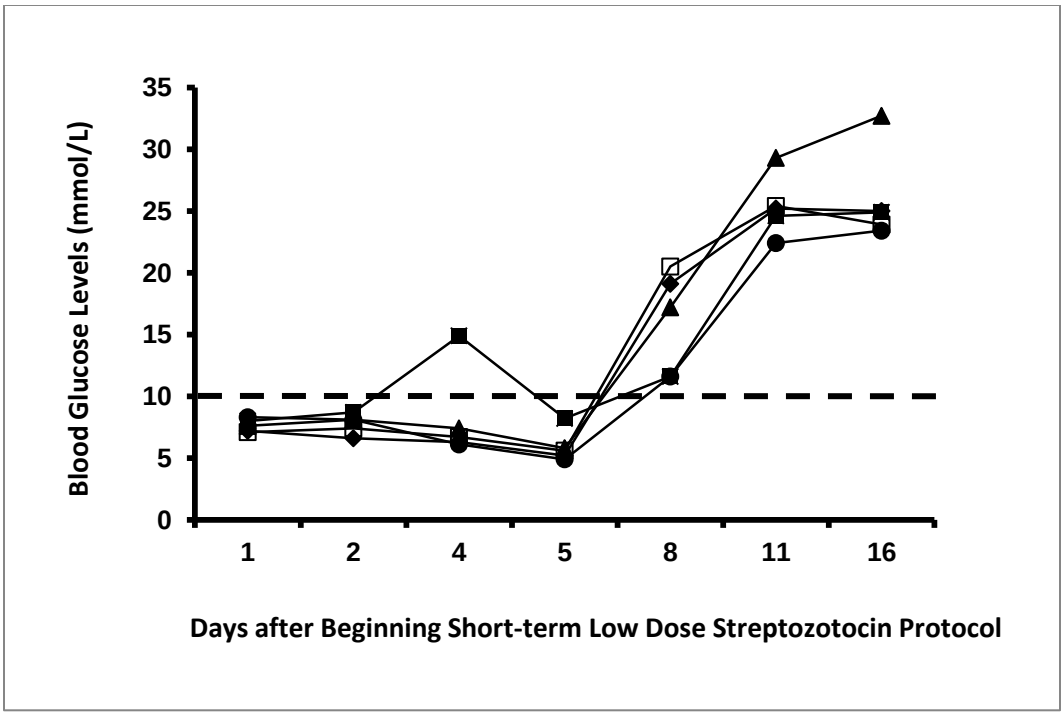

Figure A-1: Blood glucose levels of 10-12 week old NOD mice treated with a multiple low dose streptozotocin regimen. Mice were injected with 50 mg/kg of streptozotocin on 5 consecutive days beginning on day 1. Blood glucose levels were monitored daily to identify the onset of diabetes.

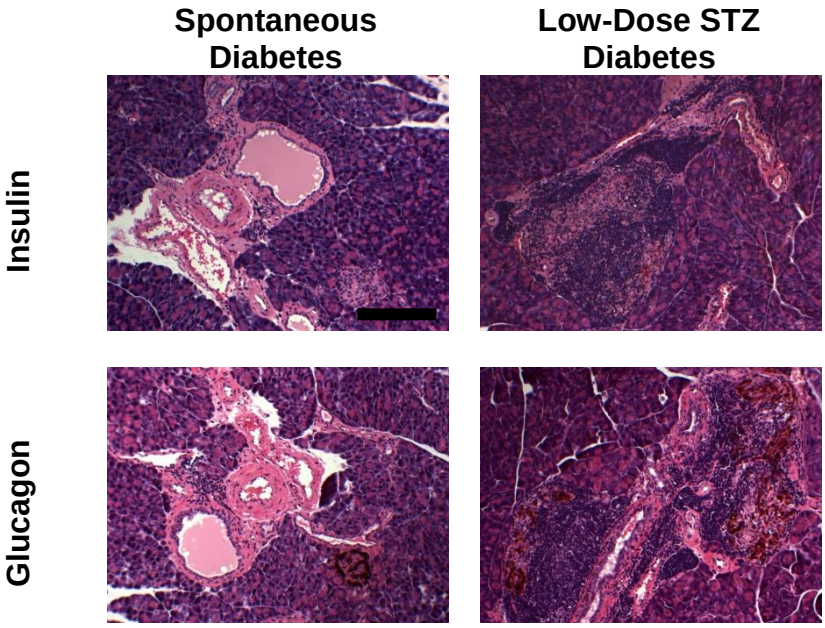

Figure A-2: Immunohistochemical analysis of pancreata from spontaneously diabetic NOD mice and NOD mice induced with diabetes by multiple low dose injections of streptozotocin. Brown staining indicates the presence of insulin and glucagon, respectively. The scale bar represents 400 µm.

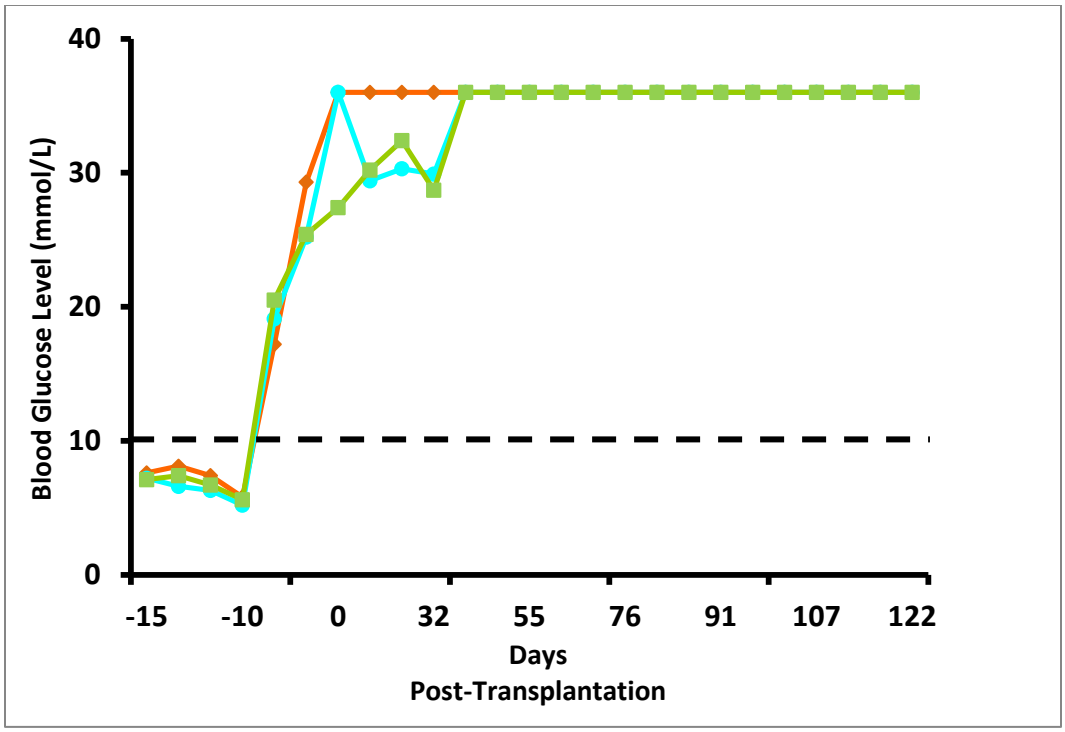

Figure A-3: Blood glucose levels of diabetic NOD mice, induced by multiple low dose injections of streptozotocin, co-transplanted with NPI and SC combined with anti-LFA-1 mAb treatment.